Unraveling fibrous dysplasia/ McCune-Albright syndrome

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PhD thesis, Leiden University, The Netherlands

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The research described in this thesis was conducted at the Department of Orthopaedic Surgery and the Department of Internal Medicine, Division of Endocrinology at the Leiden University Medical Center.

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Chapter 1

General introduction
and thesis outline

Introduction

Background and etiology

Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is a rare, benign disorder presenting with a broad clinical spectrum due to postzygotic, mosaic, gain-of-function mutations in the GNAS gene, encoding the alpha-subunit of the stimulatory G protein (Gsα)[1]. Inheritance has not been described in humans, implying lethality in case of germline transmission[2]. GNAS mutations may occur in pluripotent cells in each of the 3 germ lines, resulting in a complex array of possible abnormalities in skeletal, endocrine, and dermal tissue. The phenotype of FD/MAS depends on the timing and location of the mutation, on mutational load, viability of mutated cells and epigenetic modifiers[1, 3, 4].

Clinical presentation

Hallmark of the disease is the development of fibrous, dysplastic bone lesions with decreased mechanical bone strength, localized in one skeletal site (monostotic fibrous dysplasia [MFD]) or in several bones (polyostotic fibrous dysplasia [PFD])[5]. Approximately 80% of cases present with MFD. Disease severity ranges from asymptomatic lesions, diagnosed incidentally, to extensive debilitation due to pain, recurrent fractures or deformity[6]. When occurring in endocrine tissue, the mutation leads to hormone overproduction clinically manifesting as e.g. precocious puberty, hyperthyroidism, hypercortisolism, or growth hormone excess, while dermal mutations induce 'café-au-lait' hyperpigmented skin macules. McCune-Albright syndrome is diagnosed when fibrous dysplasia of bone is present with at least one extraskeletal feature or in the presence of two extraskeletal features[7]. MAS is diagnosed in 3% of all patients and represents the most severe subtype of the disorders with functional disability and significant morbidity. The official term for the disorder, FD/MAS, includes the entire spectrum of disease with all subtypes of FD/MAS. No scientific data exist on the prevalence of the disease, although FD has been estimated to account for 5-7% of benign bone tumors[8].

Pathophysiology of skeletal FD

GNAS mutations in skeletal tissue are affecting bone marrow stromal cells (BMSC) and induce an intricate cascade of constitutive Gsα signaling, overproduction of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) and c-fos/c-jun, with downregulation of sclerostin and overexpression of the Wnt/β-cathenin pathway. This leads to excessive reproduction of fibroblast-like progenitor cells and impaired differentiation and maturation of BMSC into mature osteoblasts and mature hematopoetic cells. Eventually, highly prolifera-

tive immature, woven, fibrous bone incorporates the marrow space with loss of normal marrow features including hematopoiesis and adipogenesis[1, 9-12].

Apart from bone formation, also bone resorption is stimulated, due to up-regulation of the RANK/RANKL pathway and of cytokines including IL-6 and TNF-alpha. Additionally, patients with high disease burden may show increased production of the phosphaturic hormone fibroblast growth factor 23 (FGF-23), which worsens the already poor mineralization and increases the risk for FD-related complications including fractures and deformity[13-16]. Strikingly, the mosaic nature is not only observed within the patient's skeleton but also within individual FD-lesions, as mixtures of normal and FD cells are frequently detected, with mutated cells only proliferating in the presence of healthy cells[17-19]. Histologically curved, irregular, thin trabeculae are seen with undermineralized, woven bone, with active bone formation and resorption, with hypercellular marrow fibrosis (figure 1)[12]. Although FD may occur in any bone, a predilection is observed for craniofacial bones, femur, humerus, ribs and spine.

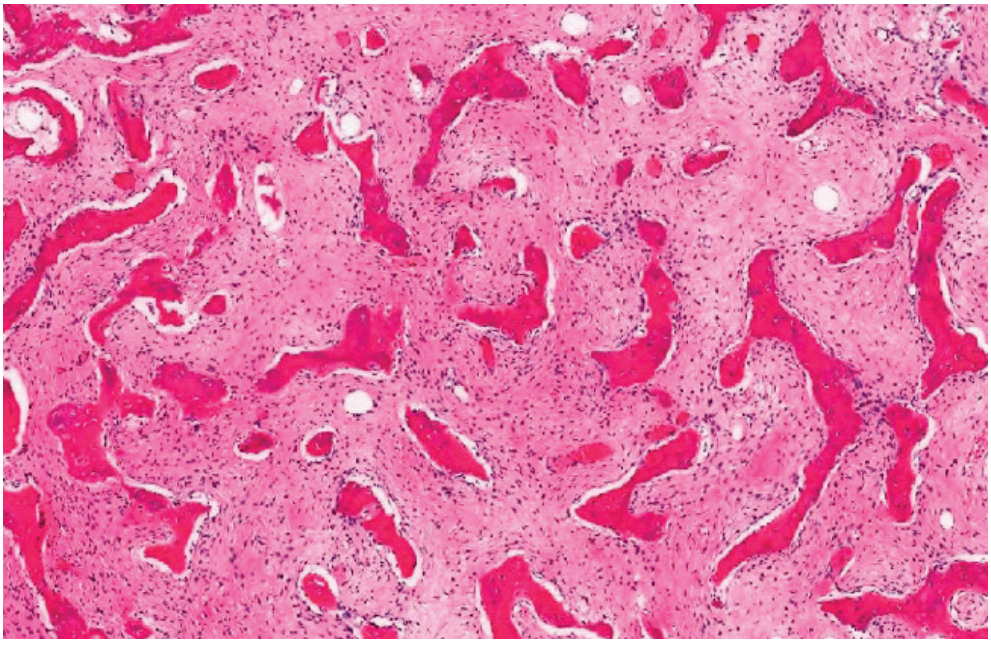

Figure 1. Histological image of FD. Curved, thin, irregular trabecula with woven bone are seen in with cell-rich fibrous stoma. Adapted from: Fibrous dysplasia, Bone & joints, by K. Wakeman and J. Mantila. [20]

Systemically, the ongoing bone turnover may lead to high serum levels of bone formation markers alkaline phosphatase and P1NP and high levels of resorption marker beta-crosslaps[7]. Clinically FD lesions may induce pain and decreased mechanical bone strength may manifest in fractures and deformities. Mainly the proximal femur is susceptible for varus deformity, also termed Shepherd's Crook deformity (figure 2), given the shape and weight bearing forces, and lesions of the lower extremity in general may sustain fractures and pose severe difficulties in ambulation[8, 21]. Spinal FD may lead to scoliosis[22], while craniofacial lesions may facial asymmetry, proptosis, or nerve compression with audiovisual impairments when expansive[8]. Most lesions appear at young age and >90% of clinically significant lesions are diagnosed before the age of 15[23]. Some case reports have described malignant transformation of FD into osteosarcoma, fibrosarcoma or chondrosarcoma[24, 25]. This should be considered in the presence of new or focal pain, pain evolving from mechanical to chronic, or new expansions in previous quiescent lesions[7, 24]. It is however very rare and more often, disease activity diminishes with age[23, 26].

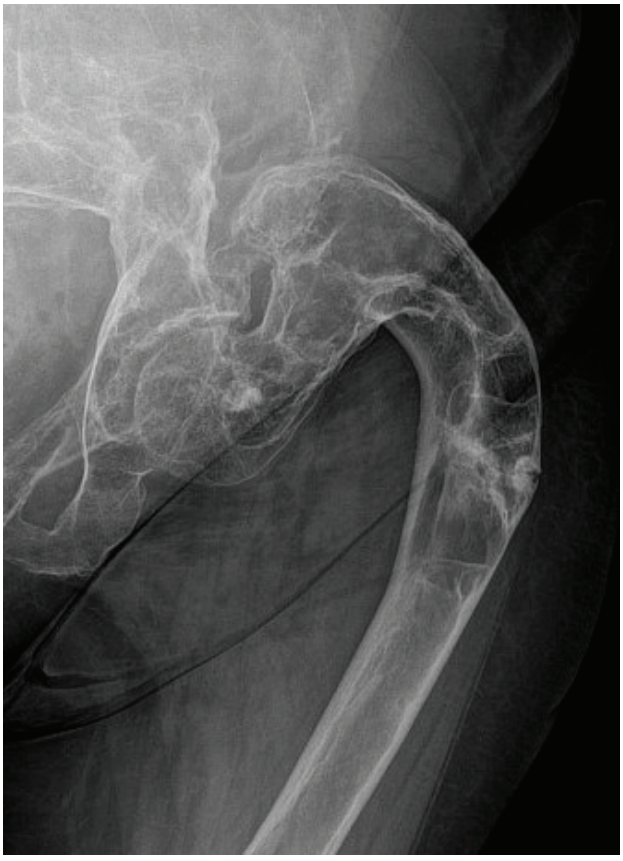

Figure 2. Large, cystic FD lesion in the proximal femur with Shepherds crook deformity.[27]

Extra-skeletal manifestations of FD/MAS

Hyperpigmented skin macules, previously called café-au-lait spots, may be one of the earliest signs of MAS directly after birth. They harbor the GNAS mutation and follow a characteristic distribution, often alongside but not crossing the midline of the body. Similarly to bone, in endocrine tissues GNAS mutations lead to an overactive state with hormonal overproduction which usually present in childhood. In patients with MAS, autonomous hypergonadism is the most common endocrinopathy and may lead to precocious puberty, in female patients to ovarian cysts, and in males to macro-orchidism or microlithiasis[28, 29]. Hyperthyroidism may be present in more than 50% of patients with MAS and may be accompanied with thyroid lesions[28]. Pituitary disease may present as growth hormone excess (GHE) and/or prolactin excess. Both GHE and hyperthyroidism are associated with enhanced skeletal morbidity, and with precocious puberty, all three endocrinopathies may accelerate growth in early childhood [22, 28]. However final length may be impaired due to early closure of growth plates and skeletal deformities. Neonatal hypercortisolism (Cushing's disease) is rare but possibly lethal and may require bilateral adrenalectomy [28, 30]. Furthermore, a wide range of cystic, benign or malignant lesions have been reported. Benign intramuscular myxomas are mainly observed in muscles overlaying affected bone and may bear the same GNAS mutation. The combination of FD with myxomas is termed Mazabraud syndrome. An increased risk for several types of (pre)malignant lesions has been described, mainly in mutation-bearing tissues or tissues with high turnover, including thyroid cancer[25, 31], cancer of the breasts and reproductive organs[25, 29, 32, 33] , gastrointestinal and hepatobiliary neoplasms, and intraductal papillary mucinous neoplasms of the pancreas[34, 35].

Psychological consequences

Several domains of quality of life are significantly affected in patients with FD/MAS. Factors demonstrated to play a role are lesion location, total disease burden, age, negative patient illness perceptions and passive coping strategies. The influence of the degree of pain on quality of life has been ambiguous in several studies[36-40].

Diagnostic work up

Due to its rarity and heterogeneity, FD/MAS is preferably treated in tertiary referral centers. With insufficient experience, the diagnostic process might be hindered by endocrinopathies presenting merely with subclinical signs as well as a wide differential diagnosis of bone lesions mimicking monostotic FD[7]. Every patient should be asked for a detailed history of complaints, presence of nociceptive and neuropathic pain, and specifically night or mechanical pain, signs of precocious puberty, growth abnormalities, developmental delay, or of

other endocrinopathies. A detailed physical examination of the entire skeleton and skin is recommended as well as assessment of pubertal stage and of the thyroid gland. Laboratory testing should include levels of markers of phosphate and calcium homeostasis, renal profile, bone turnover markers, and if indicated markers of endocrinopathies. Radiological characterization is primarily performed with plain X-ray, where FD is usually observed as well-circumscribed lesion, with typical ground glass appearance, with cystic or sclerotic areas or both, possibly expansive with endosteal scalloping and/or (pending) fracture (figure 3). CT may be useful for anatomical characterization of craniofacial, pelvic of spinal lesions and for subtle fractures, whereas MRI may be used to differentiate between FD and other cystic lesions. With nuclear imaging, the extent of skeletal involvement may be investigated with tracer uptake identifying lesional activity (figure 3).[7] Biopsy is only required in equivocal diagnosis or suspicion of malignancy. Histological evaluation may be combined with mutation analysis, although false-negatives are likely due to the mosaic nature of lesions.

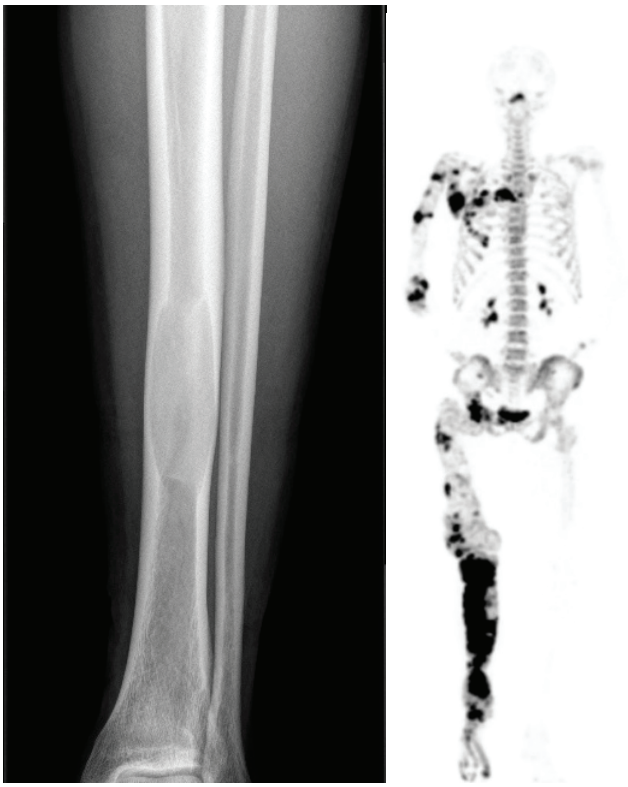

Figure 3. Imaging for FD. Left: X-ray of lower leg, with well-circumscribed, ground-glass lesion with cortical thinning. Right: Nuclear imaging ($Na^{18}F$-PET/CT) in patient with mainly left leg involvement. [27, 41]

Treatment options

Current treatments are symptomatic, to relief symptoms and prevent disease-related complications, but are unable to cure FD. General measures include promoting a healthy lifestyle with adequate dietary intake of calcium and physical therapy or orthoses if needed. Hypophosphatemia should be targeted with correction of inadequate vitamin D and calcium levels and with phosphate supplementation. Treatment of extraskeletal features is essential for bone metabolism. Oral bisphosphonates have shown no benefit for pain or skeletal complications and are not recommended[42]. Intravenous bisphosphonates, mainly pamidronate and zoledronate, may decrease pain and disease activity, although studies are not consistent and not all patients experience symptom relief[26, 43, 44]. In case of insufficient effect, denosumab may be considered. This monoclonal antibody has demonstrated a reduction in pain, bone turnover, and lesional growth rate[45-49]. Main concerns include rebound effects after therapy withdrawal and osteonecrosis of the jaw[45].

Surgery may be performed for deformity correction, stabilization of (pending) fractures, craniofacial nerve decompression, or cosmetic reasons. Surgery may be difficult because of open growth plates and expected growth in children, and because of hardware problems arising from low bone strength or severe deformity. Cortical strut grafting may be used to reinforce small lesions without fractures, with the risk for graft resorption or other complications. Curettage or cryotherapy is not recommended[50-54].

Aims of this thesis

Symptomatic FD/MAS may result in severe pain, difficulties in ambulation or audiovisual performance, decreased quality of life, and need for intensive hospital treatment. A subset of patients experience symptoms already in early childhood with life-long debilitations. Clinicians face several diagnostic and therapeutic challenges, emerging from the rarity of the disease, heterogeneity in presentation and treatment response, and complex pathophysiology with multiorgan involvement. This thesis aims to explore of the pathophysiology of FD in basic and translational research, to analyze epidemiology of FD in a registry study, and to observe the natural history of FD and treatment effects in several cohort studies.

Outline of this thesis

Part 1: Pathophysiology

Chapter 2 describes the first ex-vivo culture model for FD/MAS. This study aims to explore tissue viability and cell functioning of osteoblasts and osteoclasts in explanted surgical waste tissues of FD after 7 days of culturing. In addition, histological features of FD are assessed in comparison to control tissue, to confirm sections to be representative of FD.

In Chapter 3, serum levels of potential biomarkers RANKL, OPG, IL-6 and sclerostin and their correlations with pain, skeletal burden and treatment response are identified in patients with FD/MAS, to assess whether these markers are superior over standard markers ALP, P1NP and CTX in representing disease activity or severity and useful for clinical practice.

In Chapter 4, the link between RANKL upregulation and the increased risk for breast cancer in FD/MAS is explored by comparing RANKL expression in breast cancer specimens of patients with FD/MAS, matched control patients without FD/MAS, and pregnant patients. RANKL expression is identified by immunohistochemistry and is noted as presence of expression, positive area percentage and staining intensity. Tissue areas with RANKL expression are characterized as either breast cancer or surrounding healthy mammary tissue, to guide in hypothesizing underlying pathophysiological mechanisms.

Part 2: Disease characterization

For Chapter 5, the epidemiology of FD/MAS is evaluated in a nation-wide registry in Denmark, with data on incidence, prevalence and temporal trends re-

trieved between 1995 and 2018. Patients with monostotic disease are identified with ICD-10 code M85.0 and with polyostotic disease or MAS with code Q78.1. Incidence rates and prevalence were calculated and stratified by FD subtype (MFD versus PFD/MAS), 10-year age intervals, sex, and 5-year calendar periods.

Chapter 6 aims to elucidate the etiology of the coxa vara deformity. In children and young adults with FD located in the proximal femur, the femoral neck shaft angle is measured on all consecutive radiographs and patients with deterioration of deformity are identified. Radiographic, lesion-related risk factors as well as general, patient-related risk factors are hypothesized and their influence on progressive decrease in neck shaft angle is calculated to create a risk profile for progressive deformity.

Part 3: Treatment

Chapter 7 sets out to review the value of treatment in a multidisciplinary care pathway for FD/MAS, by comparing scores for pain measured with Brief Pain Inventory (BPI) and scores for quality of life measured with Short Form 36 (SF-36) between baseline and after 1 year of treatment in a tertiary referral center. The temporal change in scores is distinguished for newly referred patients and patients under chronic care in the trajectory, and appreciated with respect to the clinical important difference.

Chapter 8 investigates safety of discontinuation of denosumab therapy in adult patients with symptomatic disease. Data on pain and on serum bone turnover markers and calcium are established during denosumab therapy and after withdrawal. Additionally, the occurrence of potential rebound effects after withdrawal including fractures, pain flares or lesion progression are assessed.

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Part 1

Pathophysiology

Chapter 2

Human ex-vivo tissue culture model for Fibrous Dysplasia/ McCune-Albright syndrome – a proof-of-concept study

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Abstract

The complex pathophysiology of fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is incompletely elucidated and no human disease models are available to address personalized medicine. This proof-of-concept study aimed to develop a near-patient ex-vivo culture model, aiming to unravel disease mechanisms and capture individual response to medication. Five skeletal tissues of four patients with symptomatic FD/MAS were explanted. FD tissues were cultured for 0, 4 or 7 days in either normoxia or hyperoxia conditions. PCR was performed to confirm the genetic diagnose. Total number of osteocytes and number of empty lacunae were measured on HE staining. Apoptotic osteocytes were detected with a Cleaved caspase-3 (CC3) assay. Cell activity was detected by osteocalcin (OC) and alkaline phosphatase (ALP) staining for osteoblasts and tartrate-resistant acid phosphatase (TRAP) activity for osteoclasts. Histomorphometric measurements were performed on day 0, 4, and 7 of the culture in a region of interest of 1,000,000 um^2. For CC3, OC and TRAP, number of positive cells/tissue area (TA) was identified as cell count, for ALP positive area/TA as positive area percentage. PCR demonstrated R201C mutation in 3 samples, R201H in 1 sample, wild type in 1 sample. Tissue integrity remained intact after 7 days of culturing. Qualitative assessment confirmed typical features of FD compared to control. Hyperoxia conditions showed improved tissue viability and similar cell activity compared to normoxia. In hyperoxia, percentage of empty osteocyte lacunae changed from median 34.7% at baseline to 49.5% at day 7; median CC3 cell count in fibrous tissue respectively zero to 5.6 and in bone zero at all harvesting days; OC resp. 15.1 to 22.2; TRAP resp. 5.5 to 3.9; median percentage for ALP was 100% at all days. In conclusion, this 7-day culture model demonstrated sufficient cell viability and preserved cell activity and may be used for future research exploring effects of pharmaceutical agents.

Introduction

Fibrous dysplasia/McCune-Albright syndrome is a rare disorder with a prevalence of 61 per 1,000,000 persons[1]. The complex pathophysiology of FD/MAS results from a gain-of-function mutation in the GNAS locus on chromosome 20q13.3[2], mostly R201H (63.9%) or R201C (36.1%)[3]. Constitutive Gsα signaling and subsequent overproduction of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) and c-fos/c-jun in bone marrow stromal cells (BMSC) impairs differentiation into mature osteoblasts, adipocytes and hematopoietic stem cells and leads to excessive reproduction of fibroblast-like osteoprogenitor cells[2, 4, 5]. Through downregulation of sclerostin and overexpression of the Wnt/β-cathenin pathway, highly proliferative fibrous lesions develop. Fibrous tissue replaces normal bone marrow, while trabeculae consist of woven bone, irregularly shaped and poorly mineralized. This undermineralization is in some patients exacerbated by overproduction of the phosphaturic hormone fibroblast growth factor 23 (FGF-23)[5-9]. Eventually, early osteoblast differentiation markers including Runt-related transcription factor (RUNX) and alkaline phosphatase (ALP) are locally upregulated in FD tissue, late markers including sclerostin may be decreased, and mid to late markers such as osteocalcin are variable between studies, possibly depending on lesional and patient characteristics[10-15]. Additionally, coupling of bone remodeling is altered in FD/MAS by bidirectional upregulation of RANKL/RANK signaling, stimulating osteoclast function and bone resorption as well as reversibly promoting osteogenic proliferation and fibrous bone formation[11, 14, 16]. This crosstalk is additionally modified by pre-osteoblasts overexpressing cytokines including (but not limited to) interleukin 6 (IL-6), other interleukins, tumor necrosis factor (TNF-)α and Dickkopf-related protein 1 (DKK-1), vascular endothelial growth factor (VEGF), and brain-derived neurotrophic factor (BDNF) with osteogenic, inflammatory and angiogenic effects on the microenvironment[17-20]. Interestingly, somatic mosaicism is not only observed within the patients' skeleton but also in individual FD-lesions, where mixtures of mutated and normal cells are observed in variable proportions and normal cells are even required for lesion proliferation[11, 21, 22]. Due to these variations in mutational load, as well as in expression profiles and in histological characteristics, significant heterogeneity is observed between patients: pathophysiologically in lesional characteristics and activity, and clinically in location and extent of affected tissue[14, 22, 23]. Recently, a wide range of basic and translational studies have contributed to unravel the complex pathophysiology of FD/MAS and to study possible treatment targets, each with their unique strengths and limitations. Animal studies do not fully resemble human pathology and are not able to correlate histological or radiological findings with pain. A disadvantage of cellular models, both animal and human, is the difficulty to adequately model

the somatic mosaicism and microenvironment. Lastly, histological studies on explanted and immediately processed (uncultured) human FD tissue are not suitable to demonstrate temporal change or to test new therapeutic agents, since fixation at a particular moment in time prevents tissue to adapt to stimuli and comparing different samples over time may induce sampling bias[10, 24]. Yet new, effective and personalized therapies are urgently needed, especially for patients refractory to current medications. This study aimed to develop a near-patient ex-vivo culture system of FD tissue from symptomatic bone lesions of patients with FD/MAS. Over the course of 7 days of culturing tissue integrity, cell viability and cell activity was evaluated by determining apoptosis and osteoblast and osteoclast marker expression. Additionally, tissue characteristics were compared to healthy bone tissue. This study represents the first step of a patient-specific FD/MAS bone explant culture model.

Methods

Patient recruitment

Patients were recruited in the LUMC from a previously described cohort of patients following a multidisciplinary coordinated care trajectory[25]. Inclusion criteria were symptomatic and histologically confirmed FD/MAS and surgical treatment regardless of surgical site or surgical technique. Surgeries were performed according to standard procedures and skeletal surgical waste material was collected for culturing. All patients with FD/MAS signed informed consent for data and tissue collection through our Biobank Bone Remodeling and Mineralization Disorders and Ethical approval has been obtained[26]. Surgical waste material from one healthy control was anonymously used with informed consent.

Tissue harvest and culture

In this proof-of-concept study, culture methods were adapted from the model of Van de Merbel et al., who successfully established individualized treatment response in bladder and prostate cancer in an ex-vivo culturing model[27]. Immediately after explantation, surgical waste FD tissue was collected in culture medium (Minimal Essential Medium α with GlutaMAX™, Gibco, with 1% penicillin/streptomycin and 10% fetal calf serum) at room temperature. The harvested tissue of each case was divided into 24 equal pieces of approximately 1 mm^3 if the tissue was of sufficient volume. A subset of tissue was processed at day 0 for histology (n=4) and for PCR (n=4). Allel-specific reverse transcription PCR (RT-PCR) was performed to confirm the genotype in fibrous tissue according to Bianco et al., 1998[21]. A different set of tissues was cultured for 4 days in nor-

moxic conditions (n=4) or in hyperoxic conditions (n=4) or for 7 days in normoxic or hyperoxic conditions (both n=4). Tissues were cultured on nitrocellulose filter inserts (pore size of 4 μm, Corning Costar) on culture medium in 6-wells plates at a humidified incubator at 37°C in either 21% O_2 (normoxic) or in an oxygenated sealed container system in 95% O_2 (hyperoxic). Medium was replaced at day 1 and for tissues cultured for 7 days again at day 4. After culturing for 4 or 7 days, tissues were harvested and prepared for histology. Therefore for each assessment (day 0 histology and PCR, histology after culturing 4 days and 7 days in hyperoxic and normoxic conditions), 4 tissues were available for further analyses.

Histology and immunohistochemistry

Tissues were fixed in 4% paraformaldehyde for 1h at room temperature, decalcified in EDTA for several days until macroscopically sufficiently pliable, dehydrated by incubation in increasing concentrations of ethanol and cleared in xylene. Tissues were embedded in paraffin, sectioned in slices of 5 um, deparaffinated and rehydrated. Hematoxylin/Eosin (HE) stain was used for general histology; to visualize tissue structure and integrity and to assess cell morphology, specifically the presence of osteocyte nuclei in osteocyte lacunae. Of all 4 tissues per measurement, a slide was stained with HE and the 2 tissues with the highest quality were selected to proceed with the following assays. Apoptotic cells were assessed by cleaved caspase-3 (CC3) expression. Cellular activity was determined by osteocalcin (OC) expression for mature osteoblasts and by tartrate-resistant acid phosphatase (TRAP) activity for osteoclasts. Alkaline phosphatase (ALP) expression was used to determine activity of fibroblast-like osteoprogenitor cells, in order to evaluate disease activity and to confirm samples to be representative for FD/MAS. Differences between FD and control tissue were assessed in all assays.

OC, CC3 and ALP expression was detected using immunohistochemistry. First, endogenous peroxidase was quenched in 0,3% hydrogen peroxide 10 min. Heat-induced antigen retrieval was performed for CC3 and ALP in 10% citrate buffer (Sigma C9999, pH 6.0) 30 min at 100°C and non-specific binding sites were blocked in 5% Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS) for 30 min at room temperature. For OC, no antigen retrieval was used and 2% normal human serum (NHS) was added to BSA/PBS. Next, for CC3 staining, sections were incubated with Rabbit anti-human Cleaved caspase-3 (Cell Signaling, 9661L) 1:500 in 1% PBSA with isotype rabbit IgG overnight at 4°C, followed by DAKO EnVision™ anti-rabbit 30 min at room temperature, NovaRed (Vector, SK-4800) 5-7 min and Hematoxylin 1:4 for 45 sec. For osteocalcin, mouse monoclonal osteocalcin antibody (R&D systems, MAB1419) and IgG isotype were used in a concentration of 0.08 ug/ml in 2% NHS/1% BSA/0,1%Tween/

PBS. Sections were incubated 2 hours at room temperature, overnight at 4°C, following DAKO EnVision™ anti-mouse (Dako K4001) with 5% NHS 30 min at room temperature. Staining was visualized with DAB 8 min and Hematoxylin 1:4 30 sec. ALP was detected by rabbit monoclonal anti-alkaline phosphatase (Abcam, ab108337) 0.1%/1% BSA/0,1%Tween/PBS with isotype rabbit IgG for negative control. Sections were incubated overnight, followed by secondary antibody DAKO EnVision™ anti-rabbit (K4003) 30 min, both at room temperature. For color development, sections were incubated with DAKO liquid DAB+ Substrate chromogen system (K3468) 10 min and Mayers Hematoxylin (Merck 1.09249) 1:4 30 sec. For enzymatic TRAP staining, slides were incubated with Acid Phosphatase kit (Sigma-Aldrich, 387A) for 1h at 37°C protected from light and counterstained with 0.01% Light Green staining solution (Sigma-Aldrich, L1886) 1 min. Polarized light microscopy was used to assess spatial organization of collagen fibers in bone (lamellar versus woven bone) in HE stained sections, for all other experiments light microscopy was used. Tissues were scanned with Philips Digital Pathology Solutions (PHILIPS Electronics) and uploaded to QuPath 0.3.2 (Queen's University Belfast) for qualitative and quantitative analysis[28].

Qualitative assessment and histomorphometry

Both of the stained sections per time point (day 0, 4 or 7) per condition (hyperoxic or normoxic) per patient were visually inspected, and for each measurement the single section with the highest quality was selected to proceed with histomorphometric analyses. In HE stained sections, the number of osteocyte lacunae without visible nucleus and the total number of osteocyte lacunae were identified, to calculate the percentage of empty lacunae. The remaining osteocytes with visible nucleus and vital morphology were assumed to be viable. In CC3, OC, TRAP and ALP stained sections, the total area (TA) was measured as the reference area of the region of interest (ROI), as was the bone area, in 1,000,000 um^2. For CC3, individual cells with cytoplasmic expression of CC3 were identified and counted for both fibrous marrow and for trabecular bone as number of positive cells/TA. Likewise, OC and TRAP cell count was measured to describe cell activity. For OC, individual osteoblasts with cytoplasmic expression were counted and expressed as number of OC positive osteoblasts/TA of fibrous marrow. Number of osteoclasts was determined by counting multinucleated cells with cytoplasmic TRAP-expression adjacent to bone matrix/TA of trabecular bone. ALP was abundantly expressed in the fibrous marrow, hence cell activity was measured as percentage of ALP positive area/TA of fibrous marrow. Lastly, disease-specific characteristics were assessed qualitatively. HE-stained sections were used to evaluate differences in tissue structure between FD and control tissue at baseline and to visualize woven and lamellar bone at baseline, to confirm the diagnosis of FD. Similarly, the extent and localization

of CC3, osteocalcin, TRAP and ALP within the area of interest were compared between FD and control sections, with ALP as most important indicator of FD and marker of disease activity. Qualitative assessments were performed by two investigators and histomorphometry measurements were manually conducted by one investigator.

Statistical analysis

Baseline characteristics of patients were assessed including age, sex, location of lesions, reason for surgery, clinical, biochemical and radiological features, and treatment history. Data were pseudonymized for FD/MAS patients, while data of the healthy control patient were anonymously used without identifiable data. Histomorphometry results were summarized as median, given the non-normal distribution of the data, and compared between normoxic and hyperoxic conditions and between time points (day 0, 4, 7). Because of the low number of samples and the proof-of-concept design of the study, no statistical tests were performed. After extraction of the data from QuPath, all analyses were performed with SPSS version 25.0 (IBM, Armonk, NY, USA).

Results

Baseline characteristics

Five FD specimens were cultured (table 1). Three patients suffered from craniofacial disease without extraskeletal features and were operated for deformity and pain (n=2) or optic nerve compression (n=1). Two cultures were performed of lesional tissue of the same patient (4 and 5), when corrective osteotomy was performed on both femurs several months apart. This patient presented with severe skeletal disease, growth hormone excess, precocious puberty and hyperpigmented skin macules. RT-PCR revealed wild type in case 1, R201H mutation in case 3, and R201C in samples 2, 4 and 5.

Tissue integrity and cell viability

Tissue structure and integrity was qualitatively assessed in HE stained sections and compared over time (day 0, 4 and 7) and between normoxic and hyperoxic culture conditions. In all experiments tissue integrity remained intact over time, indicated by preserved cellular morphology and tissue architecture. The percentage of empty osteocyte lacunae as a marker for osteocyte death increased most between day 0 and day 4 and stabilized between day 4 and 7 (table 2, figure 1A), with large differences between cases. At baseline the median percentage of empty lacunae was 34.7%, with hyperoxia providing lower osteocyte death compared to normoxia over the culture period (respectively median of 50.8% at day

4 and 49.5% at day 7, and 66.2% at day 4 and 66.0% at day 7). CC3 expression was absent in either bone or fibrous stroma at baseline. In fibrous tissue, median cell count rose to 5.6 after 7 days of hyperoxic culturing and to 9.0 for normoxic culturing, while in bone tissue, the median remained 0 over the duration of the culture (range 0-12.2) (table 2, figure 1B). Hence, current apoptosis was limited in fibrous tissue and absent in bone tissue, with equal results in normoxic and hyperoxic culture conditions.

Table 1. Baseline characteristics

Case	Age (y), sex	Type of FD	Tissue site	Surgical indication	Surgical procedure	Biochemistry (reference)	Therapy	PCR
1	58, F	Monostotic craniofacial	Maxilla	Deformity, pain	Resection/ remodellation	All normal	1x zoledronate 5 mg 10 months prior to surgery	Wild type
2	16, F	Polyostotic craniofacial	Sphenoid bone	Compression optic nerve	Nerve decompression	ALP normal P1NP 64 (<59) CTX normal	APD 30 mg 2y prior to surgery, zoledronate 4 mg 2 w prior to surgery	R201C
3	26, M	Monostotic craniofacial	Maxilla	Deformity, pain	Remodellation	ALP normal P1NP 170 (<59) CTX normal	No	R201H
4	M, 15	Polyostotic, MAS	Femur left	Deformity, disability, pain	Corrective osteotomy + osteosynthesis	ALP 1669 (<390) P1NP 2400 (<1524) CTX normal	No medical therapy, multiple corrective surgeries	R201C
5	M, 16	Polyostotic, MAS	Femur right	Deformity, disability, pain	Corrective osteotomy + osteosynthesis	ALP 1669 (<390) P1NP 2400 (<1524) CTX normal	No medical therapy, multiple corrective surgeries	R201C

Cell activity

Since the percentage of empty lacunae was lower in hyperoxic compared to normoxic conditions with equal apoptosis in both conditions, viability of tissues cultured in hyperoxia was considered superior and therefore OC assays were only performed on these tissues. Even after 7 days of culturing, expression of osteocalcin and TRAP was observed. Cell count of OC-positive osteoblasts was highly variable between slides and ranged from 3 to 151 (table 2, figure 2). In all individual specimens, OC was most abundantly expressed at day 4, and the

median cell count of 15.1 at day 0 raised to 51.8 at day 4 and receded to 22.2 at day 7. Cell count of TRAP-positive osteoclasts was variable between tissues and in some specimens no expression was observed (table 2, figure 3). The median cell count of 5.5 at day 0 decreased to 3.9 after 7 days in hyperoxia and to 1.9 in normoxia. Median percentage of ALP expression was 100% at baseline (range 96.4-100%), which persisted after 7 days of hyperoxic and normoxic culture conditions to respectively 99.3% (range 71.6-100%) and 100% (range 90.1-100%) (table 2, figure 4).

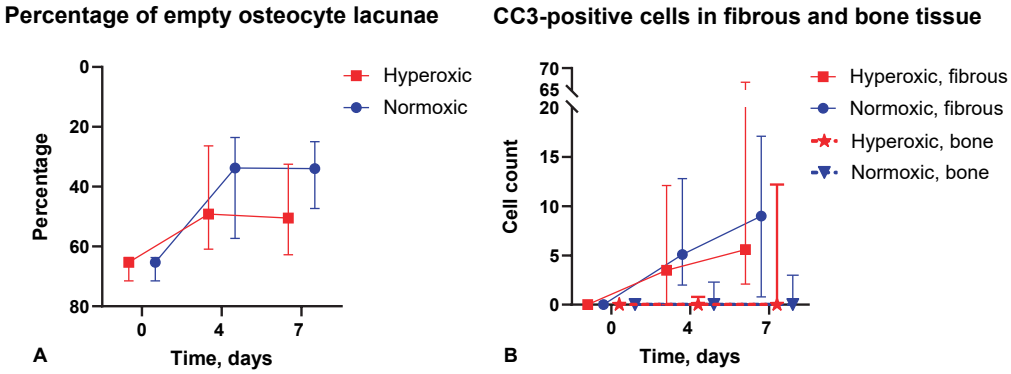

Figure 1. Percentage of empty osteocyte lacunae on total lacunae in HE stain (A) and cell count (n/1^6um^2) of positive cells in fibrous marrow and bone tissue in CC3 stain (B). Median with range is presented per culture condition (hyperoxic and normoxic) over time (day 0, 4 and 7).

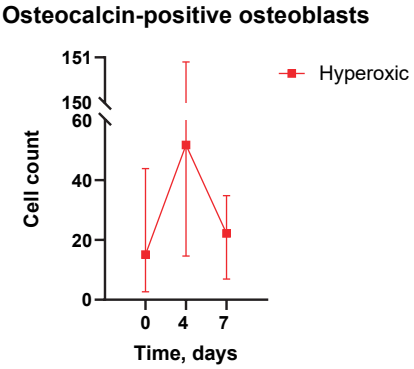

Figure 2. Cell count (n/1^6um^2) of positive cells in fibrous marrow in osteocalcin stain. Median with range is presented for hyperoxic culturing over time (day 0, 4 and 7).

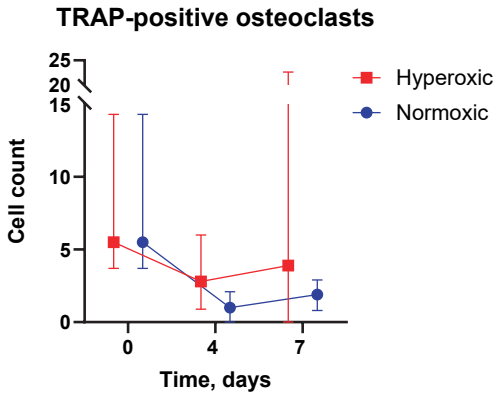

Figure 3. Cell count (n/1^6um^2) of positive cells on bone tissue in TRAP stain.
Median with range is presented per culture condition (hyperoxic and normoxic) over time (day 0, 4 and 7).

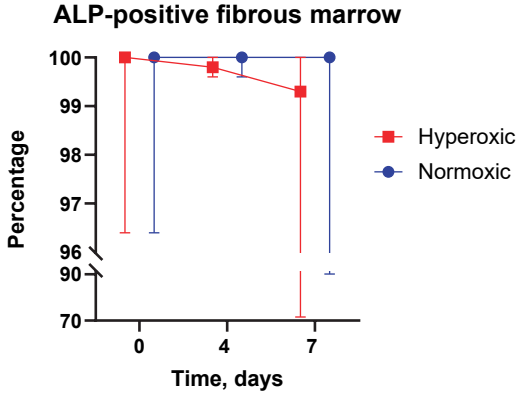

Figure 4. Positive area percentage in ALP stain.
Median with range is presented per culture condition (hyperoxic and normoxic) over time (day 0, 4 and 7).

Disease-specific characteristics

HE stained sections at baseline under polarized light demonstrated a mixture of woven bone and lamellar bone in FD case 1, 4 and 5, with occasionally both types of bone observed within the same trabecula (figure 5). Tissues 2 and 3 presented with even 80-100% woven bone. Healthy control tissue demonstrated 100% lamellar bone with normal bone marrow (figure 5). HE staining also confirmed other typical histopathological features of FD in all patients: fibrous stroma with abundant fibroblast-like osteoprogenitor cells, and thin and irregular bony trabeculae (figure 6). ALP was extensively expressed in all FD-specimens throughout the entire area of fibrous stroma, although most intense

adjacent to bone, whereas the healthy control expressed ALP in a lower intensity, clustered in certain areas in the marrow and along the bone surface (figure 7). All samples except for 1 outlier (case 2, day 7 hyperoxic culturing) exhibited a positive area percentage of nearly 100%, implying highly active FD lesions with abundant fibroblast-like osteoprogenitor cells. Osteocalcin-expressing mature osteoblasts were in healthy bone present along trabecular bone ridges, but in FD tissue scattered through the fibrous marrow, with variable expression between cases, but in general vastly lower than ALP expression. TRAP-positive osteoclasts were both in FD and in healthy specimens observed along the edge of trabecular bone and no difference was seen in morphology or quantity.

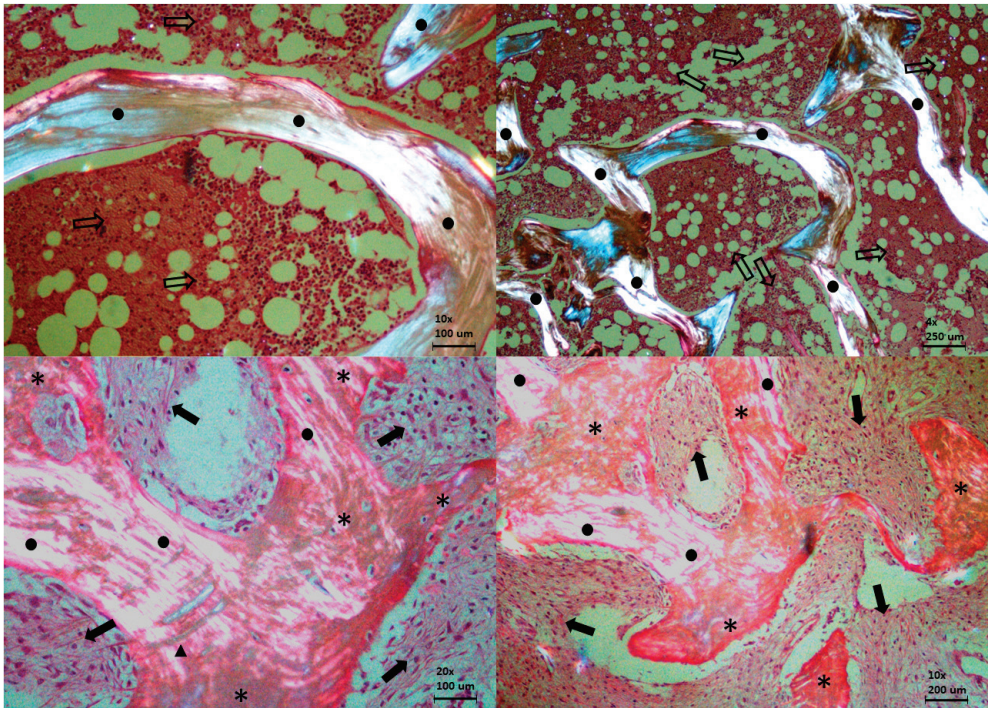

Figure 5. Spatial organization collagen fibers in polarized light.
Upper row: healthy control, 100% lamellar bone. *Lower row:* specimen 5, mixed woven and lamellar bone.
Dots: lamellar bone. *Asterisks:* woven bone. *Open arrows:* normal bone marrow. *Closed arrows:* fibrous marrow with fibroblast-like osteoprogenitor cells.

Since the abundant expression of ALP in all tissues, no correlation could be recognized with serum levels. However, serum ALP and P1NP were highest in samples 4 and 5 (of the same patient), while these tissues demonstrated least osteocalcin staining, except for one outlier of case 5 after day 4. Likewise, case 1 and 2 showed most tissue expression of OC with normal to insignificant in-

creases in serum levels of ALP and P1NP. Because of the limited expression of TRAP, the correlation between histological osteoclast activity and serum markers of bone resorption or with previous use of antiresorptive therapy could not be assessed.

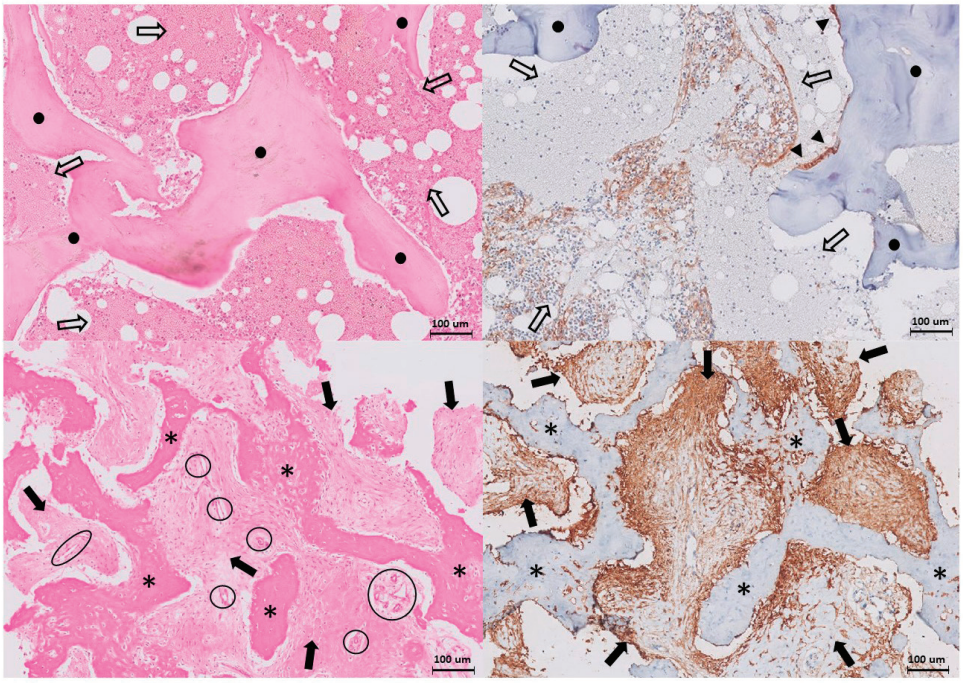

Figure 6. Healthy and FD bone in HE and ALP stain, light microscopy.
Upper row: healthy control, *left:* HE stain, *right:* alkaline phosphatase stain, brown color indicating expression. *Lower row:* specimen 2, *left:* HE stain, *right:* alkaline phosphatase stain, brown color indicating expression.
Dots: healthy trabecular bone. *Asterisks:* thin, curved, irregular bone in FD.
Open arrows: normal bone marrow. *Closed arrows:* fibrous marrow with fibroblast-like osteoprogenitor cells. *Arrowheads:* mature osteoblasts, covering bone surface (absent in FD). *Open circles:* vasculature (increased in FD).

Discussion

This study set out to evaluate tissue integrity and viability, and cell activity of FD bone tissue in a 7-day ex vivo tissue culture model, to compare hyperoxic and normoxic culture conditions, and to assess disease-specific characteristics in comparison with control tissue. The median percentage of empty osteocyte lacunae was 35% immediately after dissection and 50% after 7 days of hyperoxic culturing. Accordingly 1 out of 3 osteocytes died already at the start of the

culture, with an even higher proportion after 4 days, although the observed cell death between day 4 and 7 was stable. Very few cells demonstrated CC3 expression, with median cell count of zero at baseline in both fibrous and bone tissue and after 7 days of hyperoxic culturing 5.6 for bone marrow and 0 for bone matrix. This indicates limited current apoptosis, yet a minor increase over time. The difference between considerable cell death in HE stain and low cell death in CC3 assays is due to HE visualizing all empty lacunae up to the moment of staining, while CC3 is a marker of programmed cell death, and expressed merely by cells in apoptosis at that particular moment in time. Moreover, cell activity persisted throughout the culture period in both osteocalcin and TRAP-staining after 7 days of culturing. Mature osteoblasts may even seem to proliferate with an increase in osteocalcin expression from median cell count of 15 to 51 between day 0 and day 4, although this may be due to random variation, since after 7 days cell count regressed to baseline levels. Presence of TRAP-positive osteoclasts seemed to decline slightly over time, with median cell count of 5.5 at baseline and 3.9 after 7 days of hyperoxic culturing. However, due to scarceness of osteoclasts, their presence of can be confirmed yet caution is required in interpreting cell count. Since ALP is considered as a marker for immature osteoblasts, cell activity of immature osteoblasts remained intact during culturing with nearly 100% of fibrous marrow positive for ALP on all harvesting days. Taken together, these findings imply adequate cell viability and preserved cell activity when culturing up to seven days, sufficient to proceed with the next step of testing current and new drugs, resulting in the possibility to develop a personalized therapeutic approach for each individual patient.

Additionally, this study evaluated disease characteristics and differences to healthy control in freshly isolated, non-cultured tissue. RT-PCR confirmed the R201 and R201H mutations in four out of five samples. These proportions are comparable with a previous study describing a prevalence of *GNAS* mutations of 74%, due to, among other reasons, the somatic mosaicism of FD/MAS[29]. In all patients thin, irregular trabecular bone with hypercellular stroma was observed. Polarized light microscopy demonstrated woven bone in FD tissue, mixed with lamellar bone even within one trabecula, which is consistent with literature and reflects the mosaic nature of the disease as well as the high rate of bone formation. Abundant and intense ALP staining was seen in the entire area of the fibrous marrow, compared to limited expression in specific areas in healthy tissue. This been described in previous studies and confirms high disease activity with defective differentiation of osteoprogenitor cells into mature osteoblasts[13, 30]. OC expression was variable between FD patients, as has been linked to severity of fibrosis and rate of cellular differentiation[13, 31, 32], and scattered through fibrous marrow, in contrast with expression limited to bone-adjacent areas in healthy

control tissue. TRAP-positive osteoclasts demonstrated similar morphology and localization in both FD as normal bone, although several studies have described an upregulation in FD[19, 33]. However, due to limited osteoclasts in both tissues and no quantitative assessment of cellular density in control tissue, no formal comparison can be drawn. Overall, these results confirm that the model used tissues representative of FD and allows the exploration of FD-related disease mechanisms and differences between FD and healthy bone.

Our study is the first to explore an ex-vivo culture model for human FD tissue, able to overcome several limitations of previous basic or translational studies. Various models have offered important contributions to our knowledge on pathophysiology and mainly pre-clinical animal or cellular models are an excellent opportunity to test (novel) drugs. In mice, formation of FD-like tissue has been induced by transplanting patient-derived stromal cells or transfecting the GNAS-mutation in their skeletal stem cell lineage, in order to observe GNAS downstream signaling and disease characteristics including lesion development and histological response to treatment. Newer models have been able to silence Gsα expression in a subset of BMSC to induce phenotypic mosaicism or attempted to model hormone overproduction as seen in MAS. However, to date no model has been able to capture the somatic mosaicism of human FD/MAS, which might change dynamics between FD-mutant and wildtype cells, or to fully resemble human MAS-related extraskeletal features. Both influencing therapeutic outcomes, this poses an important disadvantage. In addition, correlation to pain and other clinical parameters is impossible in animal models, and a progressive interest in non-animal models of diseases is emerging for ethical reasons[9, 10, 21, 34-37]. Human preclinical, in vitro studies with Gsα-transduced healthy or isolated FD BMSC yielded valuable information on the requirement of somatic mosaicism for lesion formation, on the interplay between bone formation and resorption, and on expression of various downstream pathways or specific proteins. However, these cellular frameworks are not a replica of explanted tissues and do not incorporate the interplay between BMCS and their microenvironment[7, 11, 15]. Lastly, studies on isolated human FD tissue provided insights into microarchitecture, histochemical markers and effects of currently used treatments, but are not suitable to observe temporal change to ex-vivo administered drugs or to test new therapeutic agents which have not yet been approved for administration in humans[5, 6, 14, 16, 22, 30, 38]. As the model in this study used explanted, viable FD tissue, it is able to appreciate the mosaicism, microenvironment and influence of endocrinopathies as naturally occurring in the study subject, although detecting the local influence of hormone disturbances was outside the scope of this study.

Methodological aspects investigated in this study were oxygen concentration and timing of harvesting. Culturing in hyperoxic versus normoxic conditions resulted in a lower percentage of empty osteocyte lacuna and therefore superior osteocyte viability was presumed in hyperoxia, while osteoblast and osteoclast activity appeared similar in both conditions. As FD tissue has been demonstrated to be highly vascularized[24, 39], hyperoxic culturing may benefit the high oxygen demand of the tissue compared to normoxic cultures. Regardless of oxygen concentration, tissue viability diminished over time. Harvesting was now performed at 4 and 7 days, while it might be considered to shorten the culture duration for future experiments and harvest tissues at 3 and 6 days to prevent deterioration, or to pursue optimization of culture conditions to prolong culturing.

The high number of death osteocytes may be considered as one the limitations of this study. Other limitations include a small number of samples, with quantitative analyses performed merely in a single section per measurement, and heterogeneity in surgical site, surgical technique and patient characteristics. Lastly, considering the pilot status of this study, a limited number of cellular markers was assessed while many more contribute to the complex pathophysiology of FD including Wnt/B-catenin, RANKL and OPG, c-fos/c-jun and FGF-23. This proof-of-concept study was able to demonstrate adequate cell viability after culturing, but to expand the culture model to drug testing, Future studies can make use of spatial biology techniques to assess the pathophysiology or treatment response at a molecular level. To effectuate cellular changes, pharmaceuticals might need a longer treatment duration than 7 days, while prolonging the culture model beyond 7 days possibly decreases tissue viability with decreased adaptive capacity. On the other hand, an important advantage of this ex-vivo model is that administration of high doses of compounds and parallel testing of multiple agents can be permitted without FDA-approval and without adverse effects for the patient. Future studies may not only address response to various treatments or dosages, but also explore heterogeneity in histopathology and response to treatment in different FD-affected skeletal sites at the tissue level.

In conclusion, this proof-of-concept study describes the first human ex-vivo culture model for FD/MAS and demonstrates sufficient tissue integrity and cell viability with intact osteoblast and osteoclasts marker expression after 7 days of culturing. This versatile model may be exploited for future research into current or novel pharmacological agents, in order to evaluate individualized treatment response and facilitate personalized therapy.

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Chapter 3

Clinical value of RANKL, OPG, IL-6 and sclerostin as biomarkers for fibrous dysplasia/McCune-Albright syndrome

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Abstract

Background: Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is a rare genetic bone disease caused by a somatic mutation in the *GNAS* gene. Currently used bone turnover markers (BTMs) do not correlate with the clinical picture and are not useful to predict or monitor therapy success. This study assessed the correlation of RANKL, OPG, RANKL/OPG ratio, IL-6 and sclerostin with the classic BTMs alkaline phosphatase (ALP), procollagen type 1 propeptide (P1NP) and beta crosslaps (CTX), with pain, skeletal burden score (SBS) and response to bisphosphonate or denosumab treatment.

Methods: Ninety-six serum samples of adult patients >18 years of age with any subtype of FD/MAS were included from the biobank facility of the Leiden University Medical Center, Center for Bone Quality between 2015 and 2021. Standard laboratory assessments were assessed as part of usual care. The concentrations of potential biomarkers RANKL, OPG, sclerostin, IL-6 were analyzed. Data on FD/MAS subtype, age, pain, treatment history and treatment response were retrieved from the electronic patient files. Baseline characteristics were summarized by descriptive statistics. Correlations of the concentrations of the potential biomarkers with classic bone turnover markers, SBS and pain scores were cross-sectionally assessed by Spearman rank order correlation. Correction for multiple testing was performed by Benjamini and Hochberg False Discovery Rate. A sensitivity analyses was performed by excluding patients with SBS below 15 and patients using antiresorptive medication at the time of blood withdrawal or within the wash-out period. In patients treated with bisphosphonates or denosumab after blood withdrawal, pre-treatment concentrations were compared in patients with and without therapy response by Mann Whitney U test.

Results: The median age of the patients was 41.2 (Q1-Q3 25.9-52.2) years, 62.5% was female. Median SBS was 2.5 (Q1-Q3 0.5-7.8). RANKL level correlated weakly with ALP (Spearman rho 0.309, p=0.004, n=84), but not with P1NP or CTX. The RANKL/OPG ratio, OPG, IL-6 and sclerostin did not correlate with ALP, P1NP or CTX. None of the potential biomarkers correlated with SBS, pain score or pain level. Results of the sensitivity analyses were comparable. Pre-treatment biomarker levels were similar in patients with and without improvement in pain scores following bisphosphonate therapy. Pre-treatment RANKL and sclerostin were comparable between patients with and without improvement in pain scores after denosumab therapy. Pre-treatment IL-6 level and the RANKL/OPG ratio seemed to be higher in patients with response to denosumab (IL-6: median 0.64 (Q1-Q3 0.53-0.74) pg/mL, n=6, RANKL/OPG: median 0.062 (Q1-Q3 0.016-

0.331), n=5) compared to patients without response (IL-6: median 0.35 (0.20-0.54) pg/mL, n=5, RANKL/OPG: 0.027 (0.024-0.046), n=4). Pre-treatment IL-6 correlated with the improvement in maximum pain scores (rho 0.962, p<0.001, n=9) and average pain scores (rho 0.895, p=0.001, n=9) reported during denosumab therapy.

Conclusion: Increased concentrations of RANKL, IL-6, sclerostin and of the RANKL/OPG ratio do not indicate severity of FD/MAS, as no correlation was observed of these potential biomarkers with the classic BTMs and SBS. Biomarker levels did not correlate with pain and had no value in predicting bisphosphonate treatment response. These biomarkers are not superior over the currently used methods of assessing ALP, P1NP and CTX or evaluating SBS to establish disease extent or activity and provide no reliable results. Yet, possibly pre-treatment IL-6 and the RANKL/OPG ratio may have some predictive value for clinical response to denosumab. Therefore, studies investigating disease activity and treatment response should include lesional imaging and patient-reported outcome measures.

Introduction

Fibrous dysplasia is caused by a mutation in the *GNAS* gene. Due to the somatic mosaicism there is a broad clinical spectrum[1]. Hallmark of the disease is the development of fibrous, dysplastic bone lesions with abnormal bone turnover and inadequate mineralization, which may induce pain, nerve compression, fractures or malformations[2, 3]. These lesions may affect one bone in monostotic fibrous dysplasia (MFD) or multiple sites in polyostotic disease (PFD). When accompanied by at least 2 extraskeletal manifestations like hyperpigmented skin macules, hormone overproduction or benign tumors, it is termed as McCune-Albright syndrome (MAS)[4]. To date predicting disease activity and progression has been difficult and disease burden, as measured by skeletal burden score[5], does not seem to be predictive[6]. Theoretically the downstream factors from the *GNAS* mutation could serve as a marker for disease activity. The GNAS mutation is located on chromosome 20q13.3 and encoding the stimulatory alpha subunit of the G-protein (Gsα), alters hydrolysis of GTP to GDP[1, 7] and induces elevated levels of cyclic adenosine monophosphate (cAMP) and protein kinase A phosphorylation[1]. Physiologically, the increase in cAMP lowers sclerostin levels and leads to overexpression of factors of the Wnt signaling pathway and β-catenin by osteoblast progenitors. As a result, in FD/MAS this causes increased bone turnover, which may be reflected by increased markers of bone formation (alkaline phosphatase (ALP), osteocalcin, procollagen type 1 propeptide (P1NP)) and of bone resorption (beta crosslaps (CTX)). Together with insufficient mineralization, this leads to immature, woven bone[3, 8]. In addition, mutated osteoblasts overexpress receptor activator of nuclear factor κ-B ligand (RANKL)[9] and interleukin-6 (IL-6)[10, 11]. Currently disease activity is biochemically assessed by measuring the bone turnover markers (BTMs) ALP, osteocalcin, P1NP and beta crosslaps (CTX)[4]. However, these markers have several limitations in the management of FD: they do not correlate with pain[12] and correlate with skeletal burden score (SBS) in some studies but not all[5, 13, 14]. Moreover, a recent longitudinal study suggested an age-related decline in BTMs, regardless of antiresorptive treatment[12]. These limitations underline the need for biomarkers with the potential to predict and monitor treatment success and to accelerate the development of new therapeutic approaches. A previous study showed that RANKL and its decoy receptor osteoprotegerin (OPG) were systemically increased in patients with FD/MAS when compared to controls[15]. RANKL and OPG levels and the RANKL/OPG ratio correlated with SBS in a combined cohort of patients and healthy controls[15]. RANKL-inhibition has provided promising results, but the predictive value of RANKL levels for therapy success has not been investigated. Likewise treatment targeting IL-6 has been conducted, although systemic levels of IL-6 have not been evaluated in FD/MAS. Therefore, this study

aimed to explore systemic levels of RANKL, OPG, and IL-6 and to assess the association with disease activity and extent, pain and treatment response. Moreover, this study focused on sclerostin, a key bone regulator, which inhibits bone formation, suppresses mineralization and stimulates RANKL expression and bone resorption[16]. Research on the sclerostin/Wnt pathway in FD/MAS is scarce. Some patients are described to exhibit gene upregulation of components of the Wnt signaling pathway[17, 18] and in a mice model of FD, diminished expression of sclerostin was observed by immunohistochemistry, although mRNA expression was comparable to controls[19]. In summary, this study investigated the role of RANKL, OPG, IL-6 and sclerostin as potential biomarkers. Their clinical significance is evaluated by exploring the association between serum concentrations and SBS, classic BTMs, pain and response to antiresorptive therapy.

Methods

Subjects

In this cohort study 96 serum samples were collected from a IRB approved biobank[20]. All samples were retrieved from patients ≥18 years of age who visited the outpatient clinic of the Center for Bone Quality, Department of Endocrinology of the Leiden University Medical Center in the Netherlands between 2015 and 2021 and retrieved a definitive diagnosis of any subtype of FD/MAS. All samples were taken before the afternoon with a maximum processing time of 4 hours[20]. This study was approved by the Medical Ethics Committee and scientific biobank committee[20]. A subset of patients was included in previous studies[14, 21-24], data for the present study were generated originally. At the time of serum withdrawal, 65 patients (67.7%) were therapy naïve, 17 patients (17.7%) had previously received bone-modulating treatment but underwent a minimum of 6 months wash-out, 14 patients (14.6%) were under active treatment.

Clinical parameters

The electronic patient file was consulted to assess type FD/MAS, age, results of routine assessments of ALP, P1NP and CTX, presence of pain and pain score at the time of serum withdrawal. For pain assessment, the Brief Pain Inventory was used[25]. This questionnaire investigates pain severity, where patients score their maximum pain, least pain, pain on average and pain right now. The range is 0-10, higher scores reflect more pain. We considered the items maximum pain and average pain most relevant for our research and excluded other domains. Next, we classified pain scores into pain levels, where score 0 was categorized as zero pain, score 1-3 as mild pain, score 4-6 as moderate pain, and ≥7 as severe pain. Additionally, the medical history was reviewed for history of treatment

with denosumab or bisphosphonates either before or after serum withdrawal, and for therapy response in terms of pain, bone turnover markers. Treatment status during blood withdrawal was established as on or off active treatment, with a 6-month wash-out period for bisphosphonates and 3-months wash-out for denosumab. Skeletal burden score[5] was assessed on bone scintigraphy or ^{18}F-NaF PET/CT.

Laboratory analysis

From each patient, a serum sample of 450 uL was obtained from storage at -80°C in the biobank. The concentrations of RANKL, OPG, sclerostin and IL-6 were assessed according to the manufacturer's instructions. RANKL was detected by MILLIPLEX MAP Human RANKL Magnetic Bead - Single Plex - Metabolism Assay of Merck Millipore with a Luminex Platform, intra assay variation <10%, inter assay variation <15% (reported by manufacturer). Sclerostin was assessed by SOST Human Sclerostin Kit Immunoassay of MSD, intra assay variation <15%, inter assay variation <18%. OPG was measured by Human Osteoprotegerin/TNFRSF11B DuoSet ELISA with DuoSet ELISA Ancillary Reagent Kit 2 and normal goat serum, all of R&D systems, intra and inter assay variation not reported by manufacturer. IL-6 was measured by V-plex Human IL-6 kit of MSD, intra-assay variation 4%, inter assay variation 6.4%. OPG was merely assessed in the first batch of patients in 2019 (n=57) and not performed in the samples retrieved from the biobank in 2021, of newly included patients between 2019 and 2021 (n=39), due to our preliminary results. Standard laboratory assessments were routinely performed directly after venipuncture, total ALP by automated P800 modulator system (Roche BV, Woerden, The Netherlands), P1NP and B-CTX were measured by the E-170 system (Roche BV).

Statistical analysis

Baseline characteristics were summarized by descriptive statistics: number and percentage for categorical data and mean and standard deviation for normally distributed data, otherwise median with interquartile range. Results of each potential biomarker were screened for outliers by Grubb's outlier test with a significance level of <0.05. Outliers with a Z-score>3 were indicated. Correlations were assessed by Spearman rank order correlation, as the presence of relevant outliers in all laboratory assessments precluded the use of Pearson's correlation. Correlations of the potential biomarkers RANKL, OPG, IL-6 and sclerostin with classic BTMs, SBS, and presence of pain at the time of serum withdrawal were cross-sectionally analyzed. A sensitivity analyses was performed by excluding patients with SBS below 15 (to investigate whether associations were affected by disease severity) and excluding patients using antiresorptive medication at the time of blood withdrawal or within the wash-out period (to assess whether

associations were affected by use of antiresorptives). Serum concentrations of the potential biomarkers were compared in patients with and without therapy response after bisphosphonate or denosumab therapy by Mann Whitney U test. This analysis was performed in the subgroup of patients off therapy during blood withdrawal and starting therapy after withdrawal. Correction for multiple testing was performed for all statistical tests (correlations and analyses of group differences, n=26) by Benjamini and Hochberg False Discovery Rate.

Results

Baseline characteristics

Ninety-six patients were included with median age 41.2 (Q1-Q3 25.9-52.2) years, 62.5% female (table 1). Monostotic disease was present in 53 patients (55.2%), polyostotic involvement in 43 patients (44.8%). An additional diagnosis of MAS was established in 14 patients (20.8%). Median skeletal burden score was 2.5 (Q1-Q3 0.5-7.8). Median serum concentration of RANKL was 33.0 (Q1-Q3 19.5-62.5) pg/mL, of OPG 1093 (949-1478) pg/mL, of IL-6 0.342 (0.145-0.595) pg/mL, of sclerostin 0.185 (0.150-0.220) ng/mL. The median RANKL/OPG ratio was 0.024 (0.014-0.046). Grubb's outlier test was significant for all potential biomarkers, indicating at least one outlier. The number of outliers with Z-score >3.0 was 2 for RANKL (Z-score 3.1 and 7.1), 1 for OPG (Z-score 4.9), 1 for IL-6 (Z-sore 8.6), 1 for sclerostin (Z-score 3.4), and 1 for the RANKL/OPG ratio (Z-score 5.7).

Correlation of biomarkers with classic bone turnover markers

RANKL correlated weakly with ALP levels (Spearman rho 0.309, p=0.004, n=84), but not with P1NP (rho: 0.149, p=0.173, n=85) or with CTX (rho: −0.077, p=0.483, n=85) (table 2). OPG did not correlate with ALP, P1NP or CTX (rho: -0.057, p=0.676, rho: - 0.034, p=0.804, rho: - 0.127, p=0.346 respectively) (table 2). Initially the RANKL/OPG ratio did correlate weakly with ALP (rho 0.365, p=0.014, n=45) but not after correction for multiple testing. RANKL/OPG ratio did not correlate with P1NP or CTX (rho: 0.162, p=0.282, rho: -0.155, p=0.304 resp.) (table 2). Likewise initially IL-6 correlated with ALP (rho 0.245, p=0.019, n=92), but not after correction for multiple testing. IL-6 did not correlate with P1NP or CTX (table 2). Sclerostin did not correlate with any of the BTMs (table 2).

Correlation with skeletal burden score

None of the biomarkers correlated with SBS (table 2). Of note, also none of the classic bone turnover markers (ALP, P1NP, CTX) correlated with SBS (data not shown).

Table 1. Baseline characteristics

Total	**N=96**
Age years, median (Q1-Q3)	41.2 (25.9 - 52.2)
Sex, female (n, %)	60 (62.5%)
Type of FD [a] (n, %)	
MFD (non-CFD)	53 (55.2%)
PFD	43 (44.8%)
MAS	14 (20.8%)
Skeletal Burden Score median (Q1-Q3)	2.5 (0.5 – 7.8)
Use of bone modulating medication (lifetime)	
Bisphosphonates	64 (66.7%)
Denosumab	13 (13.5%)
Tocilizumab	1 (0.01%)
At time of serum withdrawal	12 (12.5%)
Bisphosphonates	1 (0.01%)
Denosumab	1 (0.01%)
Tocilizumab	
FD-pain present at baseline	50 (52.1)

MFD: monostotic fibrous dysplasia, PFD: polyostotic FD, MAS: McCune-Albright syndrome

Table 2. Correlation biomarkers with standard markers of bone turnover and SBS

		ALP	**P1NP**	**CTX**	**SBS**
RANKL	Coefficient	0.309	0.149	-0.077	0.116
	Significance	0.004*	0.173	0.483	0.294
	N	84	85	85	84
OPG	Coefficient	-0.057	-0.034	-0.127	-0.078
	Significance	0.676	0.804	0.346	0.564
	N	56	57	57	57
RANKL/OPG	Coefficient	0.365	0.162	-0.155	0.170
	Significance	0.014	0.282	0.304	0.259
	N	45	46	46	46
IL-6	Coefficient	0.245	-0.086	-0.139	0.133
	Significance	0.019	0.413	0.182	0.208
	N	92	93	93	92
Sclerostin	Coefficient	0.029	0.104	0.169	-0.141
	Significance	0.783	0.313	0.101	0.173
	N	95	96	96	95

P-values with * are statistically significant after correction for multiple testing.

Correlation with pain

RANKL, the RANKL/OPG ratio, IL-6 and sclerostin did not correlate with average pain or maximum pain score or level at the time of serum withdrawal. Table 3 shows the median serum concentrations of the biomarkers for each level of average pain. Results for maximum pain are not shown but comparable.

Table 3. Serum concentations of biomarkers for levels of average pain

		Zero pain	Mild pain	Moderate pain	Severe pain
RANKL	Median IQR (Q1-Q3) N	34 20-67 43	20 15-57 7	41 24-74 17	32 24-56 8
OPG	Median IQR (Q1-Q3) N	1171 916-1557 27	1260 862-1570 5	1135 1018-1677 10	1064 851-1187 9
RANKL/OPG	Median IQR (Q1-Q3) N	0.024 0.011-0.057 21	0.018 0.011-0.053 4	0.024 0.012-0.033 9	0.042 0.026-0.057 7
IL-6	Median IQR (Q1-Q3) N	0.346 0.128-0.535 48	0.580 0.325-0.830 7	0.315 0.150-0.640 17	0.455 0.370-0.708 10
Sclerostin	Median IQR (Q1-Q3) N	0.196 0.150-0.244 49	0.180 0.166-0.200 8	0.165 0.144-0.255 18	0.175 0.140-0.210 10

Sensitivity analyses

After excluding patients with SBS<15, the more severely affected subgroup consisted of nineteen patients. In this group, results were similar to the total group regarding the correlation between investigated biomarkers and clinical endpoints (classic bone turnover markers, SBS or pain score). Fourteen patients used bone modulating therapy at the time of blood withdrawal (14.6%) or were within 6 months prior to blood withdrawal. Exclusion of these patients from the analysis improved the correlations of RANKL with ALP and with P1NP, of RANKL/OPG ratio with ALP, and of IL-6 with ALP. However, this did not alter the (absence of) correlation between RANKL and RANKL/OPG ratio with SBS and of the (absence of) correlation between biomarkers and pain scores.

Pain response after bisphosphonate therapy

Sixty-four patients were treated with bisphosphonate therapy (66.7%). Thirty-six patients (56.3%) received their first dose after blood withdrawal, 17 patients (26.6%) had received bisphosphonate therapy prior to the blood withdrawal but underwent >6 months wash-out, of which 10 had received only therapy before the study assessments and 7 both before and after. Lastly, 11 patients (17.2%) were treated with bisphosphonates at the time of blood withdrawal. Of the 43 patients (44.8%) with treatment after but not during blood withdrawal, 4 had no pain prior to treatment, and data regarding pain response was missing or inconclusive in 3 patients. Of the 36 remaining patients, 22 patients reported improvement in pain scores following bisphosphonate therapy and 14 experienced no improvement. Patients reporting improvement in pain had similar

pretreatment RANKL levels compared to patients without improvement in pain (respectively median 24 (Q1-Q3 15-49) pg/mL, n=20, versus median 26 (17-58), n=11, Mann Whitney U: p=0.451). Similarly for the pretreatment RANKL/OPG ratio no difference was observed between groups (group with improvement: median 0.014 (Q1-Q3 0.009-0.026), n=10, versus group without improvement: median 0.019 (Q1-Q3 0.010-0.024), n=9, MWU: p=0.740, neither for IL-6 (group with improvement: 0.33 (Q1-Q3 0.13-0.65) pg/mL, n=20, group without improvement: median 0.44 (0.18-0.70) pg/mL, n=14, MWU p=0.545), neither for sclerostin (group with improvement: median 0.20 (Q1-Q3 0.15-0.25) ng/mL, n=22, group without improvement: 0.16 (Q1-Q3 0.15-0.22) ng/mL, n=14, MWU p=0.240).

Clinical response after denosumab therapy

Thirteen patients (13.5%) were treated with denosumab. Of those, one received treatment during blood withdrawal, one had received therapy prior to but not after blood withdrawal, and 11 patients used denosumab after blood withdrawal but not during (10 denosumab naive patients and 1 patient who restarted therapy after a wash-out period). Of the latter group, six patients reported an improvement in pain scores while five did not. The concentrations of the biomarkers were evaluated but a statistical comparison between groups was not possible due to the low sample size. The pre-treatment RANKL levels were comparable between the group with and without clinical improvement following denosumab therapy (respectively median 57 (Q1-Q3 19-545) pg/mL, n=5, and 33 (Q1-Q3 20-71) pg/mL, n=5). The pretreatment RANKL/OPG ratio was slightly higher in patients with improvement in pain scores (median 0.062 (Q1-Q3 0.016-0.331), n=5) than in patients with no improvement (0.027 (0.024-0.046), n=4), although the spread of the data was high. Concentrations of IL-6 seemed to be higher in patients with clinical response (median 0.64 (Q1-Q3 0.53-0.74) pg/mL, n=6) compared to patients without improvement in pain (median 0.35 (0.20-0.54) pg/mL, n=5). Sclerostin levels were median 0.21 (Q1-Q3 0.16-0.28) ng/mL in the patients with improvement in pain scores (n=6) and 0.17 (0.16-0.18) ng/mL in patients without improvement (n=5). The correlation of IL-6 and pain was assessed because of the largest difference in groups for this biomarker. IL-6 level correlated with the improvement in maximum pain scores after denosumab therapy (Spearman's rho 0.962, $p<0.001$, n=9) and with the improvement in average pain scores (rho 0.895, p=0.001, n=9).

Discussion

This study aimed to explore the value of the potential biomarkers RANKL, OPG, IL-6 and sclerostin for assessing disease activity and extent and for predicting and monitoring therapy success in patients with FD/MAS. The results suggested a weak correlation of the RANKL levels, the RANKL/OPG ratio and IL-6 levels with the ALP level, although highly variable data with multiple relevant outliers reduced the strength of this correlation. No correlations were observed between these potential biomarkers and P1NP or CTX, despite the correlations of the classic BTMs with each other. An explanation might be found in the large spread of the laboratory assessments, but nevertheless the absence of robustness of the results implies that RANKL, the RANKL/OPG ratio and IL-6 might not reflect disease activity. Similarly, OPG and sclerostin did not correlate with the BTMs. Selecting merely patients with SBS >15 did not change the results, so apparently the degree of bone remodeling assessed by the BTMs was not adequately captured by the potential biomarkers.

Correspondingly, no correlation was observed between the biomarkers and SBS, even after the exclusion of patients with SBS < 15 or under active FD-related treatment. These results are in contrast with the study of De Castro et al[15], which demonstrated a strong correlation between RANKL and SBS and a moderate correlation between the RANKL/OPG ratio and SBS. This difference may be explained by the considerably higher SBS and less outliers in the cohort of De Castro et al. Apart from SBS, their study provided no patient characteristics, hindering the comparison of patient and disease characteristics between both cohorts. The ELISA kit used for detecting RANKL was similar in both studies, but one major difference was the study population: healthy subjects were included in the study of De Castro, and analyzed with a SBS set at 0, and more severely affected patients with FD/MAS compared to our study. This methodological consideration could explain the differences in the observed correlations between RANKL or the RANKL/OPG ratio and SBS in both studies. Regardless the explanation, these differences confirm that validating previous results in a less severely affected cohort is important before drawing conclusion on the clinical utility of the data. Results of both studies combined suggest that increased concentrations of RANKL or the RANKL/OPG ratio may indicate presence but not severity of FD/MAS, and therefore provide no clinical usefulness over the currently used approach of assessing BTMs with the SBS on nuclear imaging. In addition, in our study none of the assessed biomarkers correlated with average or maximum pain. Ideally, a biomarker would aid in discriminating FD-related pain from pain arising from other sources and consequently in allocating the most suitable treatment. The identification of such markers is hampered by

the lack of a gold standard to distinguish between causes of pain in FD/MAS and the complex pain mechanisms with both nociceptive and neuropathic components involved[26]. Previous research postulated increased osteoclast activity as a possible cause for pain in FD/MAS, with IL-6 and RANKL as key osteoclastic factors[11, 26, 27], possibly combined with neuropathic changes induced by IL-6, proposed because of the role of IL-6 in bone cancer pain, neuropathic and inflammatory pain[28]. Although elucidating the complex pain mechanisms of FD/MAS in general is of utmost importance, the absence of any correlation between RANKL, IL-6 and pain in our cohort argues against further research on this topic in patients with mild to moderate disease severity. This is intensified by our observation that none of the biomarkers was associated with clinical response after bisphosphonate treatment, as the pre-treatment concentrations of the biomarkers were similar between patients with and without pain response after therapy and therefore not predictive for therapy success. A heterogenous clinical and biochemical response has been described in multiple studies on bisphosphonates, but the reasons for this remain unknown[14, 29-33]. The limited treatment response in some patients, combined with emerged results on RANKL overexpression[9, 15], has led to research on RANKL-inhibition in FD/MAS. Mice studies demonstrated halted lesion growth and improved mineralization within lesions after therapy[34, 35] and in open-label, uncontrolled human studies with the RANKL-inhibitor Denosumab, registered for i.a. osteoporosis, a reduction in pain, BTMs, lesion size and lesion activity was observed[21, 22, 36]. Similarly, IL-6 inhibition has gained attention in recent years, resulting from studies which detected overexpression of IL-6 in vitro in FD, with a glucocorticoid-induced decline in IL-6 and bone resorption[10, 11, 37]. A clinical study into the IL-6 inhibitor tocilizumab for patients with FD/MAS did not show benefits for pain or BTMs after therapy in general, although a subset of patients reported a remarkable decrease in pain[38]. Collectively all therapeutic studies in patients with FD/MAS have shown a heterogenous clinical and biochemical response in humans, despite the clear role of RANKL and IL-6 in preclinical studies. None of the studies assessed pre-treatment RANKL and IL-6 levels, and this had led to our hypothesis that high concentrations of RANKL or IL-6 might be predictive for therapy response. Yet, neither RANKL, the RANKL/OPG ratio, IL-6 or sclerostin was associated with bisphosphonate treatment response. These observations dismiss any clinical value of these biomarkers in predicting success of therapy with bisphosphonates over classic BTMs, which are likewise not suitable for this purpose[14, 21, 22, 29, 30]. Possibly RANKL, the RANKL/OPG ratio and IL-6 might have some predictive value for clinical response of denosumab, as levels appeared to be higher in patients with adequate response, but the sample size of the subgroup of patients who had received denosumab is too limited to draw firm conclusions. Further research is needed to explore this association, and place-

bo-controlled studies on denosumab therapy will provide useful information on the effect on bone turnover markers and pain. Researchers may well consider adding assessments of RANKL and IL-6 in these future studies, to evaluate the predictive value of these biomarkers for treatment success.

A limitation of this study is the heterogenous patient population. Serum samples were withdrawn upon hospital intake in most patients, but a subset of patients (n=14) had received previous antiresorptive treatment or was using antiresorptive therapy during the study procedures. This limitation was partially addressed by the sensitivity analysis of excluding patients under active treatment at time of biomarker assessment, which yielded similar results as in the entire population, as well as by assessing the predictive value of the potential biomarkers only in patients who received bisphosphonates or denosumab after but not during blood withdrawal. Lastly, OPG levels were measured merely in 56 patients, constituting the first batch of samples enquired from the biobank. . The analyses for RANKL, IL-6 and sclerostin were also performed in the second set of patients, the newly included patients in the biobank between 2019 and 2021, which were retrieved in 2021 to increase sample size, due to preliminary results combined with limited resources, OPG measurements were not performed in this subgroup. Strengths of this study include the overall large sample size for a disorder of this rarity and the exploratory nature of the study. Additionally, this study is the first to assess systemic IL-6 and sclerostin values in patients with FD/MAS. Another important aspect of our study is the validation of results of previous studies on RANKL and OPG, as this demonstrated that results may lack generalizability when conducted in small cohorts subjected to selection bias.

So far, it seems that trying to extrapolate disease activity from systemic markers is not the correct approach and we must conclude that methods that investigate lesion activity like ^{18}F-NaF PET/CT scan are so far the only methods showing a correlation between pain, bone markers and treatment response. Therefore, studies investigating treatment responses should include lesional imaging in order to discuss disease activity, as markers of bone turnover or cytokines related to bone turnover do not provide reliable results. Altogether, this study investigated the clinical usefulness of potential biomarkers: RANKL, OPG, their ratio, IL-6 and sclerostin in an exploratory setting. The assessed biomarkers do not have superior discriminative value compared to the currently used methods in assessing disease extent or activity. In addition, these biomarkers did not correlate with pain and had no value in predicting treatment response of bisphosphonate therapy. Future research may address the role of RANKL and IL-6 in predicting or monitoring treatment success of denosumab, and may

shine further light on the complex pathophysiology of FD/MAS to identify possible new treatment targets.

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Chapter 4

Expression of RANKL in breast cancer in patients with Fibrous dysplasia/ McCune-Albright syndrome

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Abstract

Background: In fibrous dysplasia/McCune-Albright syndrome (FD/MAS), mosaic mutations in the *GNAS* gene lead to locally abnormal bone turnover. Additionally, patients with FD/MAS, particularly with thoracic lesions, have an increased risk for breast cancer. Development and progression of breast cancer has been associated with expression of Receptor Activator of NF-κB ligand (RANKL) in mammary tissue, and due to the *GNAS* mutation, RANKL is systemically increased in patients with FD/MAS. Yet it is unknown whether breast cancer in FD/MAS is also dependent on RANKL. We hypothesized that the *GNAS* mutation might induce RANKL overproduction and an oncogenic niche in mammary tissue, and examined RANKL expression in breast cancer tissue of patients with FD/MAS compared to controls.

Methods: Nine patients with FD/MAS and breast cancer were included and clinical data were retrieved. Patients were matched to controls with breast cancer without FD/MAS based on age and tumor type. Three pregnant breast cancer patients were included as positive controls. Immunohistochemical detection of RANKL was performed on formalin-fixed paraffin-embedded breast cancer specimens. Staining intensity was classified as weak, moderate or intense. The area of positive RANKL staining divided by the total ductal-lobular area was assessed (positive area percentage, PAP). Number of patients with RANKL expression was compared between FD/MAS and control group by chi-square (χ^2) test, the PAP by Mann-Whitney U test (MWU).

Results: RANKL expression was observed in 3 patients with FD/MAS (38%), mainly in healthy tissue, and none of the control patients (χ^2 p=0.055). The FD/MAS group demonstrated considerably more intense staining than the control group, comparable to positive controls. The median PAP was 0.64% (range 0.14-2.04%) in the 3 FD/MAS patients with RANKL expression, 0.01% (Q1-Q3: 0.0003-0.514%) in the entire FD/MAS group, 0.006% (Q1-Q3: 0.001-0.012%) in the control group (MWU=0.574), and 0.19% (0.08-0.32%) in the pregnant patients. All patients with FD/MAS and RANKL expression had thoracic bone lesions, but no correlation was observed between RANKL expression and presence of the *GNAS* mutation or FD disease burden.

Conclusions: The triad of a higher number of patients, higher positive area percentage and stronger intensity in the FD/MAS compared to the control group indicates that RANKL may be upregulated in mammary tissue in a subset of patients with FD/MAS, which may explain the increased risk for breast cancer, although the clinical significance remains unclear. Further research is needed

to establish risk profiles for the development of RANKL-positive breast cancer and to improve early screening and treatment.

Introduction

Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is a rare genetic bone disease with variability in phenotype and severity caused by a postzygotic, mosaic mutation in the *GNAS* gene[1]. The mutation can occur in each of the three germ lines and knows an extensive list of possibly affected tissues[2]. Most commonly affected is the skeleton, where dysplastic bone lesions may cause pain, nerve entrapment, fractures or malformations[1, 2]. Next to bone lesions, extraskeletal manifestations of the *GNAS* mutation comprise endocrinopathies in MAS or intramuscular myxomas in Mazabraud's syndrome (MZB). Moreover, several benign and malignant tumors are more frequently observed in patients with FD/MAS compared to the general population[2, 3]. A recent study from our group indicated that patients with FD/MAS have an increased risk for breast cancer at a younger age, especially if lesions affect the thoracic region[4]. In the investigated cohort 44% of the breast cancer tissues showed a *GNAS* mutation, in contrast to 1% of breast cancers in the general population. Since the mutation was not found in all cancer tissues, we aimed to explore other underlying mechanisms.

In patients with FD/MAS circulating levels of Receptor Activator of NF-κB ligand (RANKL) are 16-fold higher compared to controls[5]. A member of the tumor necrosis factor family, RANKL interacts with its receptor RANK on osteoclast progenitor cells and stimulates osteoclast differentiation and survival. RANKL may also bind to osteoprotegerin (OPG), a soluble decoy receptor competing with RANK[6]. In the last decade it has been discovered that the RANKL/RANK/OPG triad is not only involved in bone metabolism, but also in other physiological processes, such as the immune system, reproductive system and thermoregulation[7, 8]. In addition, RANKL is detected in neoplastic tissues of the prostate, breast, bone marrow, kidney, bone and liver[7-9]. Excess levels of RANKL are thought to induce hyperplasia, chemoattraction of tumor cells, neovascularization and epithelial-mesenchymal transition, promoting tumor formation and metastasis[7-12]. Specifically, the female reproductive system and bone metabolism share RANKL-dependent features. Sex hormones are major regulators of RANKL expression in bone, by osteoblasts and bone marrow stromal cells resulting in bone resorption, as well as in the breast, by hormone receptor positive (HR+) mammary epithelial cells during pregnancy and estrous cycles, resulting in proliferation, differentiation, migration, expansion and survival of mammary stem cells in order to develop functional lobulo-alveolar structures[13-17]. However, RANKL (over)expression is also involved in mammary epithelial-mesenchymal transition and in cancer development[18, 19]. OPG/RANKL/RANK expression has been demonstrated in luminal epithelial cells in lobules and ducts of normal

breast tissue, in situ tumors and infiltrating tumors, both hormone positive (HR+) and negative (HR-) types[20, 21]. In general, RANKL expression is higher in normal breast tissue compared to neoplastic tissue[21-23] and higher in pregnant women[24, 25]. RANKL overexpression has also been associated with the presence of osteoclast-like giant cells (OGC) in breast cancer[26]. High serum levels of RANKL and high RANKL/OPG ratio seem to predispose for the development of HR+[27, 28] but not HR- cancer[28], particularly in women diagnosed at older ages[28], with progression of breast cancer[27], positive lymph nodes[29], presence of disseminating tumor cells[27, 29], and osteolytic bone metastasis[29], the latter which can be attenuated by sequestering RANKL with Denosumab treatment[30-34].

These data highlight local RANKL expression as a risk factor for the development of breast cancer, next to previously known risk factors including advanced age, Caucasian race, genetic predisposition, or high exposure to progesterone/progestins in women with early menarche, late menopause, late childbearing, or hormone replacement therapy[35]. In addition high serum levels of RANKL may be associated with poor prognosis, next to young age, advanced stage, absence of hormone receptors, or presence of the HER2neu receptors[36]. We postulate that in patients with FD/MAS the *GNAS* mutation might create an oncogenic niche in mammary tissue under the influence of RANKL (over)production, and hypothesize that this interplay may link the skeletal consequences of FD/MAS to the increased breast cancer risk. To explore this relationship, we examined the expression of RANKL in breast cancer tissue of patients with FD/MAS compared to controls.

Methods

Subjects

10 Dutch patients with FD/MAS and breast cancer included in this study were described previously by Majoor et al.[4] Clinical patient data were collected on FD/MAS-related features including age of diagnosis of FD/MAS, type of FD/MAS, skeletal burden score (SBS) and localization of FD lesions, as well as on breast cancer-related characteristics including age at diagnosis, type of breast cancer, stage, type of therapy, evidence of progression and survival. Paraffin embedded pathological specimens were retrieved from the hospitals where the breast cancer surgery was performed. A control group consisted of female patients without FD/MAS, diagnosed with breast cancer in the Leiden University Medical Center (LUMC) and matched based on breast cancer type and age of diagnosis. Breast cancer tissues of 3 pregnant woman without FD/MAS were used as positive controls, since expression is increased during pregnancy[24]. The LUMC

pathology department selected cases of both control groups anonymously and provided coded paraffin embedded pathological specimens. Hence no clinical data were available for both control groups, and no data on hormone receptor status were shared by the pathology department for the purpose of this study, although these data had been gathered for clinical purposes. All patients in the FD/MAS group had given consent as previously reported[4] and approval for the present analysis was obtained by the Medical Ethics Committee (protocol numbers G16-026 and P17-136).

Immunohistochemical detection of RANKL

Hematoxylin/Eosin stained sections were screened for the presence of tumor tissue by an experienced pathologist. Of subjects with multiple available paraffin blocks, both specimens with healthy breast tissue and with tumor tissue were selected. The blocks were sectioned (5 μm) and RANKL expression was detected by immunochemistry. Sections were rehydrated and endogenous peroxidase was quenched with 0.3% H_20_2/dH_2O. Antigen retrieval was performed by incubation with 10% citrate buffer/dH_2O for 30 min at 100°C. After blocking non-specific binding sites with 5% BSA/PBS for 30 minutes, the sections were incubated overnight at 4°C with primary anti-RANKL antibody M366 (Amgen®), diluted 1:1000 in 1% BSA/0.1% Tween/PBS. Next, the sections were incubated 30 min. at room temperature with the secondary antibody DAKO EnVision™ anti-mouse with 5% normal human serum. Finally, for color development the sections were incubated with DAB or with Nova Red and counterstained with Hematoxylin. Sections of both methods (DAB and with Nova Red) were scanned and analyzed in Qupath software, v.0.2.3 (Queen's University, Belfast, Northern Ireland)[37]. The staining was consistent across both methods using DAB or Nova Red.

Specialized analysis of RANKL expression

A pathologist estimated the percentage of lesional and of healthy tissue per section and within the RANKL positive areas. The total area of ducts and lobules (both healthy and lesional) was indicated as region of interest (ROI) using the polygon annotation tool. Similarly, the region of positive cytoplasmic RANKL staining was circled. RANKL positive area is expressed as RANKL positive cytoplasmic stained region/ROI in % and is further termed positive area percentage. Intensity of RANKL expression was assessed qualitatively and was for each polygon categorized as weak (barely positive), moderate and strongly positive (very clear and intense). Quality control of the positive area percentage of RANKL was performed by a second observer, confirming good to excellent interrater reliability with an intraclass correlation coefficient of 0.958 (95% CI 0.709-0.995). In order to reduce false positivity a threshold of 0.02% was set

for the positive area percentage. Specimens with percentage area of expression below the threshold were considered negative. All specimens with RANKL expression were screened for the presence of osteoclast-like giant cells (OGC).

Statistical analysis

Clinical characteristics were summarized as number and percentage or as median (Q1-Q3). The number of patients with RANKL expression was compared between the FD/MAS group and control group by Chi Square test. Interrater reliability of the positive area percentage was calculated by intraclass correlation coefficient based on two-way random effects model, absolute agreement, single rater. The positive area percentage was calculated for each subject, summarized as median (quartile 1 – quartile 3), and compared between the FD/MAS group and control group by Mann-Whitney U test. Characteristics of FD/MAS patients with RANKL tissue expression were compared with the entire FD/MAS group, including FD/MAS disease extent, presence of extraskeletal manifestations, tumor aggressiveness and prognosis, receptor status and additional mutations.

Results

Clinical characteristics

The FD/MAS group consisted of 10 patients (table 1). Of 1 patient breast specimens were not available anymore (case 10) and this patient was excluded from the matching procedure. The remaining 9 patients were diagnosed with ductal carcinoma in situ (DCIS) (n=4, 44%), of which 1 within a papilloma, or with infiltrating ductal carcinoma (n=5, 56%) and were matched accordingly to controls. After tissue analyses (see below) one FD/MAS patient (case 9) and one control patient (case 19) appeared to have sections of insufficient quality. These patients were excluded from analyses and are shown in table 1 and 2 in grey. Of the remaining 8 patients with FD/MAS, all had polyostotic disease and in 6 patients the thoracic area was affected (75%). 3 patients had additional myxomas (38%) and 3 were diagnosed with MAS (38%). Median age at diagnosis of breast cancer was 49.5 years (range 32–54 years). Positive controls were affected with infiltrating ductal carcinoma (n=1, 33%) or DCIS (n=2, 67%). These abnormalities will be further referred to as lesional tissue.

Tissue expression

RANKL expression with a positive area percentage above the threshold of 0.02% was observed in 3/8 patients with FD/MAS (38%) (pt. no. 1, 2 and 3), as opposed to none of the controls (χ^2 p=0.055) (table 2). The observed RANKL staining was mainly located in healthy tissue. In positive controls (pregnant women) all RANKL expression was observed in normal ductal or lobular cells (figure 1a). In the FD/MAS group, the ratio of healthy to lesional tissue within the RANKL-positive area was 60:40 for case 1, 85:15 for case 2 and 95:5 for case 3 (figures 1b). RANKL staining was absent in controls (patients with breast cancer without FD/MAS) (figure 1c), in both healthy and lesional tissue. The estimated percentages of lesional tissue on the total ROI for all patients are noted in table 2. The RANKL staining in the FD/MAS group was considerably more intense than in the control group and was comparable to the positive controls: of the total area of RANKL expression in all patients with FD/MAS combined, 52% was estimated to be of weak, 31% intermediate and 17% strong intensity, whereas in the control group, the expression (although minimal, with positive area percentage below the threshold of 0.02%) was estimated as 93% weak, 7% intermediate and 1% strong intensity, and in the positive controls 30%, 43% and 27% respectively. In case 3, entire lobules were stained intensely positive (figure 1b). Such intense and focal expression was not observed in tissue of positive controls, where expression was more scattered through the healthy lobular tissue (figure 1a), nor in controls. In patients 1 and 2 with FD/MAS the expression was also scattered. In the 3 FD/MAS patients with RANKL expression the median positive area percentage was 0.64% (range 0.14-2.04%). In the entire FD/MAS group the median percentage was 0.01% (Q1-Q3 0.0003-0.514%) and in the control group 0.006% (0.001-0.012%) (p=0.574). All 3 positive controls showed convincing RANKL expression with median positive area percentage 0.19%, range 0.08-0.32% (figure 2). No OGC were found in any of the specimens with RANKL expression.

Table 1. Patient characteristics

Patient ID	Age diagnosis FD (y)	FD type and SBS	Thoracic localization FD	GNAS mutation FD	Age diagnosis breast cancer (y)	Breast cancer stage at diagnosis	Risk factors breast cancer	(history of) Additional tumors
1	<1	MAS+MZB 24.7	Yes	NA	32	DCIS	Menarche 0 y, 12 y OCP, late pregnancy 38 y	IPMN, FNH Thyroid cysts
2	49	PFD 3.3	Yes	NA	52	T2NmicM0	Late pregnancy 36 y, family +	Parathyroid adenoma, thyroid nodules
3	3	PFD NA, spine/femur	Yes	NA	37	T2N0M0	2x IVF, no pregnancy	-
4	24	PFD+MZB 16.8	Yes	R201H	54	DCIS Gr 3	Breast cysts	Multinodular goiter
5	58	PFD 16.1	Yes	R201C	50	DCIS Gr 3	30 y OCP	-
6	2	MAS 70.6	Yes	R201C	48	T2N1M0	Menarche 7 y, no children	-
7	50	MAS+MZB 15.8	No	NA	49	T2N1M0	Menarche 7 y, family +, no children, smoking	IPMN
8	56	PFD 3.6	No	NA	50	T2N1M0	NA	-
9	<1	MAS+MZB 61.0	Yes	R201C	37	DCIS Gr 2	Menarche 0 y, smoking, no children	IPMN, adrenal adenomas, ovarian cysts
10	16	PFD 27.1	Yes	R201H	52	T3N1M0	20 y OCP, Menarche 11 y	-

Abbreviations: FD: fibrous dysplasia, SBS: skeletal burden score, PFD: polyostotic fibrous dysplasia, MAS: McCune-Albright Syndrome, MZB: Mazabraud syndrome, DCIS: ductal carcinoma in situ, NA: not available, OCP: oral contraceptive pill, IVF: in vitro fertilization, IPMN: intraductal papillary mucinous neoplasm, FNH: focal nodular hyperplasia. Grey: excluded.

Table 2. Breast cancer tissue characteristics and RANKL expression

Patient ID	Group	Tumor type	% region of RANKL expression on total ROI	% lesional tissue on total ROI	Type of RANKL positive tissue (%)	Receptor status	GNAS mutation	Other genes and type of mutation
1	FD/MAS	DCIS in papilloma	0.64	70	Healthy 60% Lesional 40%	ER/PR +, Her2/neu NA	NA	NA
2	FD/MAS	Infiltrating ductal carcinoma	0.14	40	Healthy 85% Lesional 15%	ER/PR +, Her2/neu +	No	PIK3CA: H1047A
3	FD/MAS	Infiltrating ductal carcinoma	2.04	75	Healthy >95% Lesional <5%	ER/PR -, Her2/neu +	No	ERBB2: L755S PIK3CA: H1047A TP53: A248G
4	FD/MAS	DCIS	<0.02	80	-	ER/PR -, Her2/neu +	No	PIK3CA: G545G
5	FD/MAS	DCIS	<0.02	0	-	ER/PR +, Her2/neu -	Yes	GNAS: R201C
6	FD/MAS	Infiltrating ductal carcinoma	<0.02	50	-	ER/PR +, Her2/neu -	No	PIK3CA: G545L
7	FD/MAS	Infiltrating ductal carcinoma	<0.02	0	-	ER/PR +, Her2/neu -	Yes	GNAS: R201H PIK3CA: H1047A
8	FD/MAS	Infiltrating ductal carcinoma	<0.02	75	-	ER/PR +, Her2/neu NA	No	none
9	FD/MAS	DCIS	NA	NA	-	ER/PR +, Her2/neu -	Yes	GNAS: R201C AKT1: G17L
10	FD/MAS	Infiltrating ductal carcinoma	NA	NA	-	ER/PR +, .	NA	NA
11	Control	Infiltrating ductal carcinoma	<0.02	50	-			
12	Control	Infiltrating ductal carcinoma	<0.02	95	-			

Table 2. Breast cancer tissue characteristics and RANKL expression (*continued*)

Patient ID	Group	Tumor type	% region of RANKL expression on total ROI	% of lesional tissue on total ROI	Type of RANKL positive tissue (%)	Receptor status	GNAS mutation	Other genes and type of mutation
13	Control	Infiltrating ductal carcinoma	<0.02	60	-			
14	Control	Infiltrating ductal carcinoma	<0.02	80	-			
15	Control	DCIS	<0.02	75	-			
16	Control	DCIS	<0.02	60	-			
17	Control	DCIS	<0.02	50	-			
18	Control	DCIS	<0.02	90	-			
19	Control	Infiltrating ductal carcinoma	NA	NA	-			
20	Positive control	Infiltrating ductal carcinoma	0.08	70	Healthy			
21	Positive control	DCIS	0.19	40	Healthy			
22	Positive control	DCIS	0.32	20	Healthy			

Abbreviations: ROI: region of interest. NA: not available. ER: estrogen receptor, PR: progesterone receptor, Her2/neu: Human Epidermal Growth factor Receptor

Clinical characteristics related to RANKL expression

Tumor stage/aggressiveness

Two out of 3 patients (67%) with RANKL expression had breast cancer at young age (32 years and 37 years). Despite this young age, their RANKL-positive tumors were not larger than T2 and not disseminated to lymph nodes or bones. The remaining RANKL-positive patient was diagnosed with T2NmicM0 breast cancer at age 52. In the total FD/MAS group the median age at diagnosis of breast cancer was 49.5 years (Q1-Q3 40-52) and positive lymph nodes were present in 4 out of 5 patients (80%) with infiltrative tumors (table 1). Survival was 100% and no recurrence or metastases were observed.

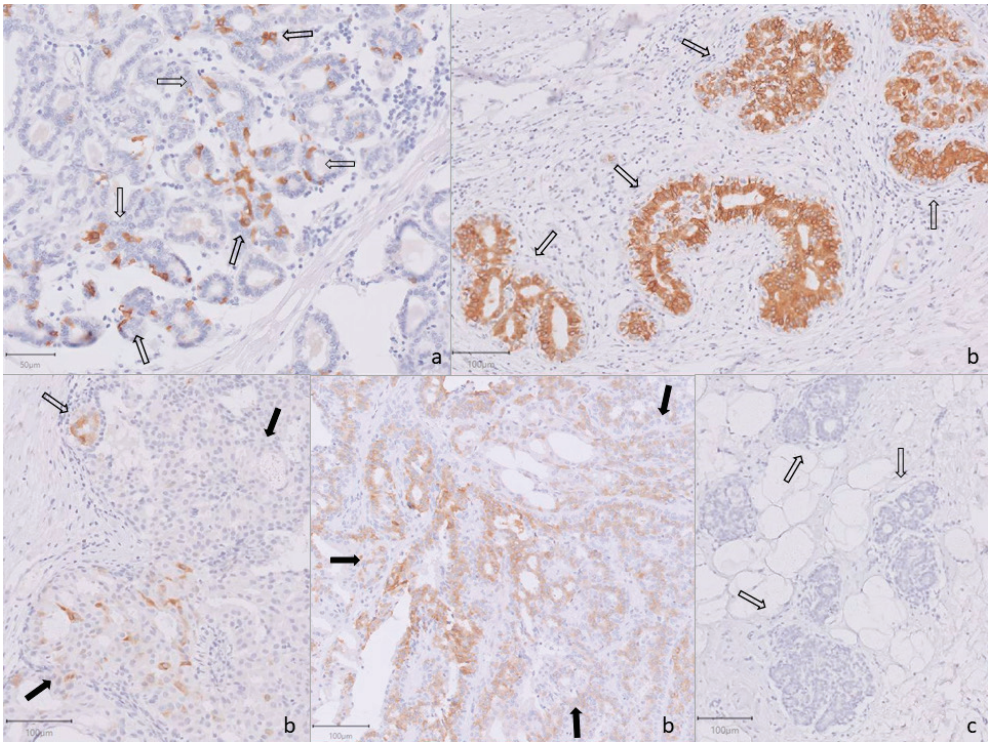

Figure 1. Immunohistochemical detection of RANKL in breast cancer tissue from a. pregnant women, b. patients with FD/MAS and c. breast cancer patients without FD/MAS. Open arrows indicate normal breast lobules and closed arrows indicate lesional breast tissue. RANKL expression is shown in brown. All sections were counterstained with haematoxillin.

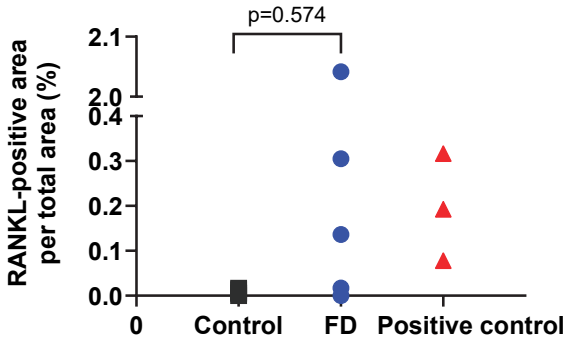

Figure 2. Percentage of RANKL-positieve area per total area in breast cancer patients without FD/MAS (control), in breast cancer patients with FD/MAS (FD) and in pregnant breast cancer patients (positive control). Patients with FD/MAS tended to have a higher percentage of RANKL-positive area per total area.

Receptor status

Hormone receptor status was positive for 2 (67%) and negative for 1 patient (33%) with RANKL expression. Her2/Neu receptor status was positive in 2 and missing in 1 patient. In the total FD/MAS group ER/PR positivity was observed in 6/8 patients (75%) and Her2/neu positivity in 3 of the 6 analyzed patients (50%) (table 2).

Genetic mutations

2 of 3 RANKL-positive patients were screened for the presence of the mutations in the breast specimens. In both patients the *GNAS* mutation was absent, but PIK3CA mutations were observed (table 2). The patient with the highest positive area percentage (pt. 3) had additional mutations in the TP53 gene and the ERBB2 gene along with Her2neu positivity. In the total FD/MAS group the *GNAS* mutation was detected in breast cancer tissue of 2/7 patients (29%) and PIK3CA mutations in 5/7 patients (671%).

FD disease extent

All 3 patients with mammary RANKL expression had FD bone lesions in the thoracic area, while in the total FD/MAS group 6/8 (75%) had thoracic lesions. Disease burden was variable: pt. 1 had a skeletal burden score of 24.7 and pt. 2 of 3.3 (table 1). In case 3 no bone scan was performed, but apart from the clinically relevant and radiographically confirmed lesions in the femur and thoracic/lumbar spine, no signs of other lesions were present. In the total FD/MAS group the median SBS was 16.1 (Q1-Q3 3.6-24.7). 1 out of 3 patients (33%) with RANKL expression was affected with extraskeletal manifestations of FD/MAS (MAS and MZB) versus 4 out of 8 patients (50%) in the entire FD/MAS group (table 1). None

of the patients had additional malignant tumors. 2 of 3 (67%) RANKL-positive patients were diagnosed with other benign or premalignant tumors: case 1 had intraductal papillary mucinous neoplasms (IPMN) of the pancreas, focal nodular hyperplasia (FNH) of the liver and thyroid cysts. Pt. 2 was diagnosed with parathyroid adenoma and thyroid nodules. Pt. 3 did not have additional tumors or cysts. In the total FD/MAS group 4 out of 8 patients (50%) had benign or premalignant tumors, specified in table 1.

Discussion

The aim of this study was to detect RANKL expression in breast cancer tissue of patients with and without FD/MAS, to explain the increased risk for breast cancer in FD/MAS. In our study, RANKL expression was detected in 38% of the patients with both FD/MAS and ductal breast cancer but in none of the control breast cancer patients. These results indicate that RANKL may be expressed in a subset of patients with both FD/MAS and breast cancer. The proportion of RANKL-positive specimens in the FD/MAS group falls within the range reported in literature, that described RANKL expression in a range of 6-78% of breast cancer tissues[14, 20, 22, 24, 26, 38-41]. This wide range may be explained by differences in methodological aspects between studies or in patient characteristics. Expression of RANKL is in both healthy and malignant tissue dependent on serum progesterone levels[18, 24, 25]. In healthy tissue RANKL expression ranges from 0-100%[18, 22, 24]. Surprisingly, no RANKL expression was detected in any of the mammary tissues from the breast cancer patients without FD/MAS, though in literature at least 6% of breast cancer cases were classified as positive for RANKL[14, 20, 22, 24, 38-41]. This discrepancy between our findings and literature might be explained by different histomorphometric measurement protocols, although most studies did not describe these details. We did observe small areas of weak and non-convincing staining in several tissues. To increase the specificity (avoid false positive findings) we used a threshold and did not include these as positive in our data, explaining the observed differences. Furthermore, the limited number of included patients may prevent detection of controls with RANKL expression.

In patients with both FD/MAS and breast cancer, merely a small area of ductal lobular tissue expressed RANKL, with a remarkably low positive area percentage ranging from 0.14 – 2.04%. This was similar to pregnant breast cancer patients and still higher than patients with breast cancer but not FD/MAS, indicating that only a subset of ductal or lobular cells express RANKL. Since this information was not mentioned in previous literature, we could not establish

whether the positive area percentage measured in our patients was deviating from other studies. A recent study on OGC in breast cancer demonstrated a percentage of positive tumor cells in specimens with RANKL expression ranging from 10% in the group with invasive cancer without OGC to 52% in the group with OGC[26]. Although different methods were used to quantify RANKL expression, the results are in line with our study, with small areas of positive cells in absence of OGC. Besides a higher positive area percentage, also the intensity of the RANKL expression was much stronger in patients with FD/MAS compared to the incidental, single-cell expression observed in controls, and comparable to the intensity observed in the positive control group of pregnant women. The pattern of overexpression of RANKL is not homogenous across patients with FD/MAS and breast cancer, a finding which might be related to the mosaicism of the mutation, although this cannot be explained with our findings. Yet, this does not detract from the conclusion that RANKL may be expressed in a subset of patients with FD/MAS. The triad of a higher number of patients, higher positive area percentage and stronger intensity in patients with FD/MAS compared to controls indicates a higher number of RANKL-positive cells as well as an increased production of RANKL by these cells, thus supports the hypothesis of RANKL overexpression in FD/MAS.

In this study, all 3 RANKL-positive patients showed additional risk factors such as: early menarche, late or no pregnancy, positive family history, or mutations in the PIK3CA, ERBB2 or TP53 genes. These risk factors in combination with the upregulation of RANKL could trigger the creation of an oncogenic niche. *GNAS* mutated breast tissue may, through paracrine signaling, alter the breast microenvironment in favor of tumor formation. In mice it has been observed that RANKL overexpression caused impaired differentiation, hyperplasia of the mammary epithelia and decreased apoptosis[42-44]. These events contribute to tumorigenesis, both in absence of oncogenic stimuli as well as in the presence of carcinogenic agents or hormonal stimulation, for which RANKL acts as mediator[14, 17, 43]. Conversely, RANKL/RANK inhibition attenuated and delayed tumor development in mice[14, 17, 27, 43, 45]. Similarly in patients with FD/MAS this oncogenic niche may allow tumors to develop in presence of other triggers as second hit, including concurrent mutations or hormonal stimulation[46]. This hypothesis could however not be extended to tissues outside of the breast, as no other malignant tumors were observed in our cohort apart from breast cancer. In addition to this paracrine effect, an endocrine effect of circulating RANKL secreted from bone cannot be ruled out, which could similarly induce an oncogenic niche[47].

Notably, our results showed that RANKL expression was observed merely in healthy tissue and only a minor proportion in lesional tissue, in papilloma or DCIS tissue, but not in ductal infiltrating components. Similarly in mammary tissue from pregnant breast cancer patients all positive cells were located in healthy tissue. In control tissue of breast cancer patients without FD/MAS neither healthy nor lesional tissue expressed RANKL, while both types of tissue were present in all sections. We had expected to observe more RANKL expression in lesional tissue, as our hypothesis was that RANKL would play a role in the development of breast cancer in patients with FD/MAS, though the results seem to be consistent with literature, where in comparative studies RANKL expression was higher in normal tissue compared to lesional tissue[22-24]. The oncogenic niche appears to be initiated by healthy RANKL-positive tissue, and the paracrine effects including (pre)malignant change seems to occur in RANKL-negative ductal or lobular cells. In this way malignant crosstalk may occur, leading to increased initiation or survival of cancer cells.

In the patients with FD/MAS the clinical implications of RANKL expression in healthy tissue for prediction, prognosis or treatment success remain unclear. In this study, RANKL expression was only observed in patients with thoracic FD lesions, although not all patients with thoracic FD lesions expressed RANKL. Surprisingly no correlation was observed between RANKL expression and the magnitude of (extra)skeletal involvement in FD/MAS or with the presence of the *GNAS* mutation. From a developmental point of view patients with FD/MAS with a more severe subtype of FD, a higher skeletal burden score, and thus more *GNAS*-mutated tissue, are expected to have a higher chance for the *GNAS* mutation as well as RANKL expression to be located in breast tissue, but our results do not support this assumption. The theory of an oncogenic niche could not account for the absence of RANKL expression in *GNAS*-positive breast cancer tissues or vice versa. RANKL expression was observed locally and scattered through the tissue and *GNAS* mutations are also known to exhibit a mosaic pattern. Since different sections were used for both the RANKL and *GNAS* analyses, the detection of simultaneous expression could have been missed. Alternatively, currently unknown mechanisms independent from RANKL may be more important contributors to breast cancer in patients with FD/MAS. In addition, no correlation was observed between RANKL expression and other clinical parameters including breast tumor type, receptor status or tumor aggressiveness. This was remarkable, as in in patients with FD/MAS increased RANKL serum levels are observed[5] and increased RANKL are associated with poor prognosis in non-FD/MAS breast cancer patients. However the absence of this association is in line with our results demonstrating expression in healthy

and not in malignant tissue and with our hypothesis that RANKL is responsible for an oncogenic niche, but for tumor progression or metastasizing.

A limitation of our study is the small sample size, a common difficulty in research into rare diseases. Moreover, data on mammary RANKL expression in patients with FD/MAS without breast cancer are lacking. Such healthy tissue was not available, but would provide valuable information. In addition, serum levels of RANKL and tissue expression of RANK or OPG were not determined. These were regarded as less relevant because of the known upregulation of skeletal RANKL in FD/MAS, but may be addressed in future research. Nonetheless, our observational study is the first to investigate possible links between FD/MAS and the development of breast cancer. Other strengths include the quantitative aspect of the measurements, high intraclass correlation coefficient, staining of both lesional and healthy breast tissue, and the use of specimens from pregnant women as positive controls, which confirmed the reliability and validity of our staining method.

Yet several questions remain unanswered. The clinical significance of mammary RANKL expression once the diagnosis of breast cancer has been established needs to be elucidated. More research is needed to establish a risk profile for the development of RANKL-positive breast cancer, which may benefit screening and treatment in an early stage. The potential role of the RANKL-inhibitor Denosumab may be addressed, a drug available for treatment of the skeletal consequences of FD/MAS[48, 49], as this might be prescribed instead of bisphosphonates for symptomatic patients with FD/MAS at risk for or diagnosed with breast cancer. Lastly, in vitro studies may elucidate the pathophysiological mechanisms underlying the development of RANKL-driven breast cancer in FD/MAS. Nevertheless, this explorative study comprises an important contribution to the understanding of the extraskeletal manifestations of FD/MAS. We conclude that RANKL expression is upregulated in healthy mammary tissue in a subset of patients with FD/MAS and breast cancer, particularly in patients with thoracic lesions, which may contribute to breast cancer formation.

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Part 2

Disease characterization

Chapter 5

Incidence and prevalence of fibrous dysplasia/McCune-Albright syndrome – a nationwide registry-based study in Denmark

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Abstract

Context: Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is a rare genetic disorder. Incidence and prevalence are not well-studied. Epidemiological research is complicated by the rarity of FD/MAS, absence of registries, heterogeneous presentation and possibly asymptomatic phenotype. FD/MAS may present with FGF23-mediated hypophosphatemia, of which the epidemiology is also unclear.

Objective: Evaluate incidence and prevalence of FD/MAS and FD/MAS-related hypophosphatemia.

Design: Cohort study based on nation-wide Danish registry from 1995-2018.

Setting: Population of Denmark.

Patients: Identified from the Danish National Patient Registry by ICD-10 codes M85.0 (monostotic FD, MFD) and Q78.1 (polyostotic FD(PFD)/MAS).

Main outcomes measures: Incidence rates and prevalence were calculated and stratified by sex, age, calendar period and diagnosis code. Cases were screened for FD-associated hypophosphatemia by diagnosis code 'disorder of mineral metabolism' (E.83) and dispatched vitamin D analogues. Measures were presented with 95% confidence intervals.

Results: 408 patients were identified, 269 with MFD (66%), 139 with PFD/MAS (34%), comparable between sexes. Incidence of FD/MAS demonstrated increasing secular trend with a rate of 3.6 per 1,000,000 person years [95%CI 2.9,4.5] in 2015-2018. Incidence peaked between age 11-20. Prevalence of FD/MAS increased over time to 61.0 [95%CI 54.6,67.4] per 1,000,000 persons in 2018. Incidence rate of MFD was 1.5-fold of PFD/MAS in the first decade, rising to 2.5-fold in the last decade. No FD/MAS cases were registered with diagnosis code or treatment for hypophosphatemia.

Conclusions: FD/MAS is rare, diagnosis peaks during adolescence without sex predominance, MFD is most prevalent. Hypophosphatemia may be underdiagnosed and undertreated, or underregistered, comparing this study to literature.

Introduction

Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is a rare disorder characterized by local replacement of bone by dysplastic tissue in a single skeletal site (monostotic FD, MFD) or in several bones (polyostotic FD, PFD). These fibrous bone lesions may lead to significant skeletal morbidity[1]. The disorder is caused by postzygotic activating mutations of the *GNAS* gene, leading to increased cAMP and abnormal cellular responses[2]. Due to the genetic mosaicism of the *GNAS* mutation, FD presents along a heterogenous clinical spectrum and bone lesions can coincide with hyperfunctioning endocrinopathies, most commonly precocious puberty or hyperthyroidism, or hyperpigmented skin macules in the McCune-Albright syndrome (MAS). Therefore the disease is referred to as FD/MAS[3]. In skeletal tissue, the maturation of osteoprogenitor cells into osteoblasts is hampered but proliferation is stimulated, causing the continuous formation of immature, woven bone[2]. In addition, phosphaturic hormone Fibroblast Growth Factor 23 (FGF23) is produced and high serum levels of FGF23 may be present in patients with FD/MAS, leading to renal phosphate wasting and hypophosphatemia[4]. The hypophosphatemia may cause osteomalacia, which is associated with pain, deformities and fractures[5-7]. Previously the incidence of hypophosphatemia in a cohort of severely affected FD patients was found to be 48% (20 of 42 cases)[4]. However, the exact incidence of hypophosphatemia in all FD/MAS patients is lacking. The same holds for the incidence of FD/MAS itself, since the monostotic subtype is often diagnosed as an incidental finding without symptoms[3] and no epidemiological studies on FD/MAS have been published. Only estimations based on regional observations are available, but these numbers are not confirmed by research studies neither replicated in different cohorts[8]. Incidence measures may provide useful information on risks by age or sex, and may aid in screening and diagnostic decision making[9]. In addition, epidemiological measures and the distribution of subtypes within FD/MAS are important for research purposes, to estimate the degree of bias and external validity (generalizability) of studies: if a study conducted in a tertiary referral center includes mainly patients with severe subtypes (PFD or MAS), the conclusions might not be applied to cohorts of less severely affected patients in smaller hospitals, but knowledge on the proportion of subtypes in FD/MAS is essential for this consideration. For these reasons, the present study was conducted to assess the incidence and prevalence of FD/MAS in Denmark with an additional interest in FD-associated hypophosphatemia, over a twenty-five year period, specified in calendar intervals, age intervals, by sex and by subtype, and to describe the demographics of the FD/MAS cohort based on the national registry data of Denmark.

Materials and methods

Data source

This nation-wide, observational cohort study used registry data of Denmark, currently having 5.9 million citizens[10]. The Danish National Health Service provides tax-supported health care, ensuring unfettered access to general practitioners and hospitals for all Danish inhabitants. Accurate linkage of all administrative and medical registries is possible at the individual level. Every citizen of Denmark is automatically enrolled in the registries without requirement of informed consent. The Danish National Patient Registry (DNPR) was established in 1977 for inpatients and outpatients were included from 1995[11]. A disease is coded as primary diagnosis when comprising the main reason for the hospital contact (although financial considerations may not be ruled out) and as secondary when supplemental to the primary diagnosis or relevant for the hospital contact, for instance underlying chronic diseases. The DNPR was used to identify patients with FD/MAS. In 1994 the 10th revision of the International Classification of Disease (ICD-10) was implemented and for these reasons the timeframe for data collection for this study was set at 01.01.1995-31.12.2018. Migration, demographic, and mortality data were available through individual-level-linkage with the Danish Civil Registration System and allowed life-long follow-up and accurate determination of person years at risk[11]. Observation period ended when the subject was no longer resident in Denmark, at death or on the 31st of December 2018, whichever occurred first. Data were also linked with the Danish National Prescriptions Registry, containing data regarding all prescription drugs dispensed at Danish community pharmacies[12]. Important to note is that for registry analyses yielding 5 or less cases per category, the exact number of patients may not be disclosed and patient demographics may not be explored for privacy reasons. The project has been approved by Statistics Denmark, the Danish Health Data Board, the Danish Medicines Agency, and Danish Data Protection Agency, with record number 2016-051-000001/1880 assigned by Arhus University.

Case definition

A subject was classified as having FD/MAS in case of at least one outpatient hospital visit encounter or inpatient hospital stay coded as monostotic fibrous dysplasia (ICD-10: M85.0), polyostotic fibrous dysplasia/McCune Albright syndrome) (ICD-10: Q78.1), or craniofacial fibrous dysplasia (ICD-10: K10.8), either as primary or secondary diagnosis. Although the latter diagnosis code also includes other fibrous lesions of the jaw, it was planned to include the diagnosis code initially and decide post-hoc whether to include or exclude the registered cases. No data exist on validity of these codes specifically, but in general the

positive predictive value of three-digit ICD-10 codes in the Danish National Patient Registry is 88%[13]. The index date was defined as the date of the first hospital contact that yielded the FD/MAS diagnosis. FD/MAS is a chronic and incurable disease and duration of disease was considered life-time.

Statistical analysis

Demographic data and epidemiological measures were calculated using R 4.2.2 and SAS software, version 9.4. Tables and graphed were designed in R 4.2.2.

Incidence

We calculated incidence rates per 1,000,000 person-years with 95% confidence intervals (CIs) using the exact Poisson distribution. Incidence data were stratified by sex, 10-year age intervals, age and calendar period, age and sex, FD subtypes (code for monostotic FD vs polyostotic FD/McCune-Albright syndrome) and calendar period, and FD subtype and age. To analyze temporal trends, incidence rates were calculated for intervals of 5 calendar years.

Prevalence

Prevalence was calculated as the ratio of persons having the disease in the calendar year. This cumulative measure included patients being incident at or before the calendar year and who remained alive. The prevalence was referenced to 1,000,000 persons alive at mid-year and the confidence interval was estimated using the normal approximation of the binomial distribution. Prevalence were stratified by sex, 10-year age intervals, age and 5-year calendar period with prevalence for every fifth year, age and sex, FD subtypes (code for monostotic FD vs polyostotic FD/McCune-Albright syndrome) and age.

Hypophosphatemia

After identifying cases with FD/MAS, all cases were screened for the presence of an encounter coded as 'Hypophosphatemia' (ICD-10 code: E83.3), and more generally coded as 'Disorders of mineral metabolism' (ICD-10: E.83). In addition, all prescriptions of alphacalcidol (ATC-code A11CC03), other active vitamin D analogues (ATC: AC11CC) and phosphate supplementation (ATC: A12CD02) to cases were identified in the Danish National Prescriptions Registry. Statistical analyses to calculate epidemiological measures of hypophosphatemia in FD/MAS were similar as stated above.

Differentiation between PFD and MAS

For patients coded with ICD-10 code Q78.1, further differentiation between disease subtypes PFD and MAS was attempted by identifying all encounters coded for MAS-related extraskeletal manifestations (endocrinopathies and café au lait

spots) and all prescribed drugs used in the treatment for endocrinopathies. All ICD-10 codes and ATC-codes are provided in the supplemental file, table A[14].

Results

The total number of unique identified patients between 1995-2018 was 408. Thirty-one patients received codes for both monostotic FD and polyostotic FD/McCune-Albright syndrome. They were regarded as having PFD/MAS and were excluded from the monostotic cohort, eventually consisting of 269 cases (65.9%). The PFD/MAS cohort included 139 cases (34.1%). In total 188 males (46%) were included and 220 females (54%). The cohort of patients with craniofacial disease consisted of 2098 cases. A high number of non-FD fibrous jaw lesions was suspected and patients with this code were therefore excluded from further analyses.

Incidence

A slight upward trend in incidence rate was observed over time: the incidence rate of 2.4 [95%CI: 1.8,3.0] per 1,000,000 person-years in 1995-1999 increased over time to reach a plateau after 2010, with a rate of 3.6 [95%CI: 2.9,4.5] in 2015-2018, comparable for men and women (table 1, figure 1). In general, incidence rates rose over time for all age categories, and in all calendar periods the highest incidence rates were observed in age category 11-20, the lowest in age over 51 years (figure 2). No shift towards earlier diagnosis in later calendar years was observed.

Table 1. Incidence per calendar period for men and women

Sex	Calendar period	Incidence rate per 1,000,000 person year	Lower CI (95%)	Upper CI (95%)
Men	1995-1999	2.486	1.711	3.492
Men	2000-2004	1.848	1.196	2.728
Men	2005-2009	2.252	1.530	3.197
Men	2010-2014	3.063	2.217	4.126
Men	2015-2018	3.923	2.861	5.249
Women	1995-1999	2.281	1.550	3.238
Women	2000-2004	2.100	1.406	3.016
Women	2005-2009	3.143	2.284	4.219
Women	2010-2014	4.425	3.400	5.661
Women	2015-2018	3.367	2.395	4.603

Incidence rates were in all age categories comparable for male and female patients, with a peak incidence in the age interval of 11 to 20 years, 5.4 [95%CI: 3.9,7.3] in males and 5.7 [95%CI: 4.1,7.6] in females (supplemental figure A, table B[14]).

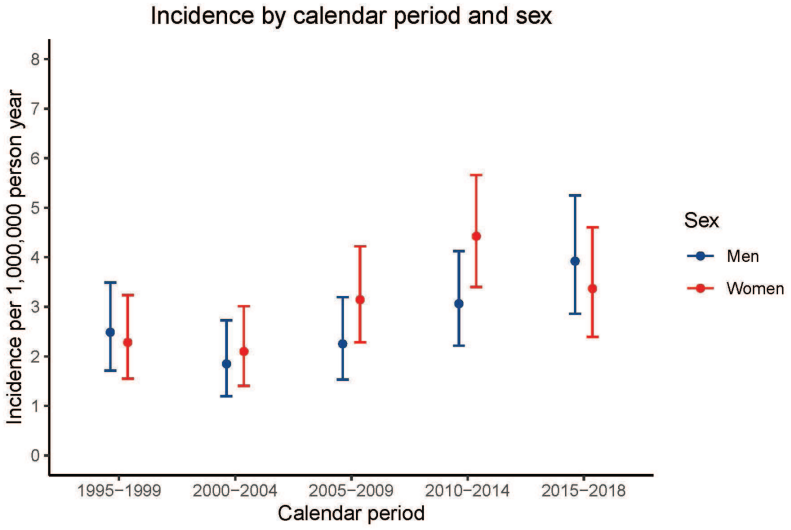

Figure 1. Incidence by calendar period and sex

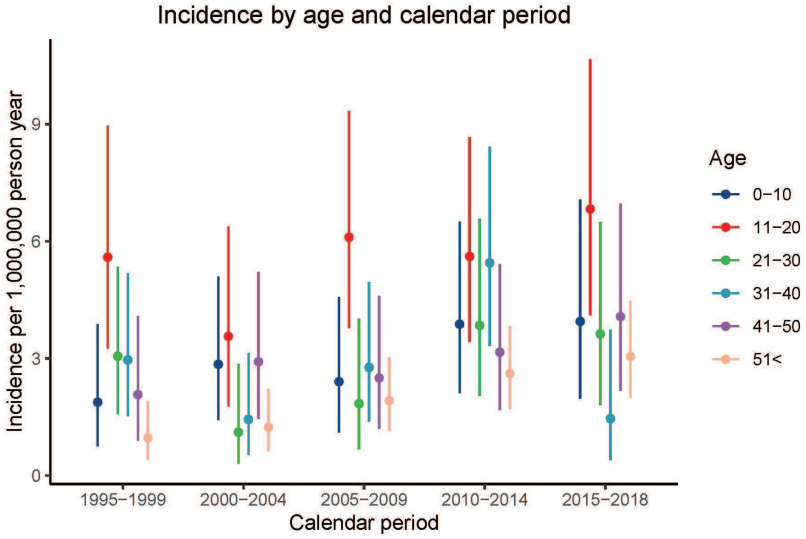

Figure 2. Incidence by age and calendar period

In 2015-2018, monostotic FD was more frequently diagnosed compared to polyostotic FD/McCune-Albright syndrome with an incidence rate of 2.6 [95%CI: 2.0,3.4] for MFD and 1.0 [95%CI: 0.7,1.6] for PFD/MAS (table 2, figure 3). For monostotic FD, this most recent incidence rate is considerably higher compared to the first calendar interval of 1995 to 1999 (1.3 [95%CI: 0.9-1.8]), whereas incidence rates were rather constant over time for polyostotic FD/McCune-Albright syndrome. In 1995-1999, the incidence rate of monostotic FD was 1.5 times the rate of polyostotic FD/McCune-Albright syndrome, in the last decade this has risen to 2.5 times, with 71.4% of diagnosis being MFD and 28.6% PFD/MAS. The peak incidence rate for monostotic FD was between ages 11 and 20 (3.8 [95%CI: 2.9-4.9]). In all other age intervals, rates were lower and comparable (table 3, figure 4). Polyostotic FD/McCune-Albright syndrome was most frequently diagnosed in the first 2 decades, with a rate of 1.5 [95%CI: 1.0-2.2] between age 0-10 and 1.8 [95%CI: 1.2-2.5] between age 11-20, and rates decreased thereafter to 0.5 [95%CI: 0.3-0.8] above age 51.

Table 2. Incidence per calendar period and ICD

Calendar period	M85.0			Q78.1		
	Incidence rate per 1,000,000 person year	Lower CI (95%)	Upper CI (95%)	Incidence rate per 1,000,000 person year	Lower CI (95%)	Upper CI (95%)
1995-1999	1.27	0.88	1.77	1.08	0.72	1.55
2000-2004	1.10	0.74	1.57	0.95	0.62	1.39
2005-2009	1.87	1.40	2.46	0.76	0.47	1.16
2010-2014	2.79	2.21	3.48	1.03	0.69	1.47
2015-2018	2.60	1.99	3.35	1.04	0.67	1.55

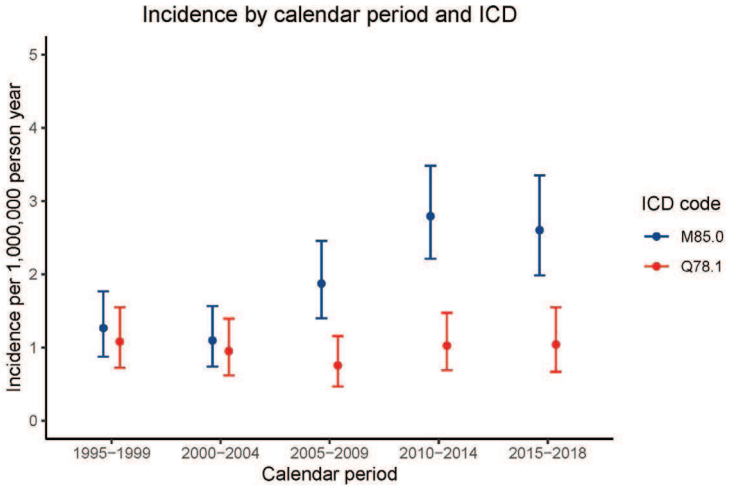

Figure 3. Incidence by calendar period and ICD

Table 3. Incidence by age and ICD

Age at incidence	M85.0			Q78.1		
	Incidence rate per 1,000,000 person year	Lower CI (95%)	Upper CI (95%)	Incidence rate per 100.000 person year	Lower CI (95%)	Upper CI (95%)
0-10	1.41	0.91	2.08	1.52	1.00	2.22
11-20	3.77	2.88	4.85	1.76	1.17	2.54
21-30	1.80	1.23	2.56	0.87	0.49	1.44
31-40	1.72	1.18	2.43	1.13	0.70	1.73
41-50	2.17	1.56	2.94	0.79	0.44	1.31
51<	1.47	1.13	1.87	0.51	0.32	0.77

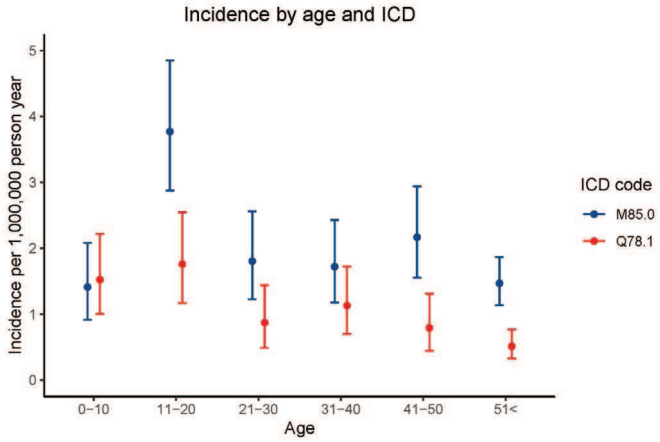

Figure 4. Incidence by age and ICD

Prevalence

Prevalence demonstrated an increasing trend, from below 0,1 per 1,000,000 persons at the start of the study period gradually inclining to 61.0 [95%CI: 54.6,67.4] in 2018 (supplemental figure B[14]). In all age stratified analyses, prevalence was lowest in age category of 0-10 years, slightly higher in category >51 years, and highest in the second to fifth decade. In 2018, prevalence was 18.9 [95%CI: 8.6-29.1] per 1,000,000 persons for age category 0-10 years and highest between age of 31-40 years (82.6 [95%CI: 61.0-104.3]) (table 4). In all age groups prevalence increased during the study period, except for age 0-10 years (figure 5).

In 2018, prevalence was in all age intervals comparable between men and women (table 5, figure 6). The prevalence of monostotic FD was 1.4 - 1.7 fold higher compared to polyostotic FD/McCune-Albright syndrome in the first 4 decades, and 2.4 – 2.9 fold higher in the two oldest age groups (supplemental table C[14]).

Table 4. Prevalence by age in 2018

Age	Prevalence per 1,000,000 person	Lower CI (95%)	Upper CI (95%)
0-10	18.9	8.6	29.1
11-20	68.4	48.8	87.9
21-30	80.0	60.1	99.9
31-40	82.6	61.0	104.3
41-50	81.1	61.1	101.2
51<	51.3	41.8	60.8

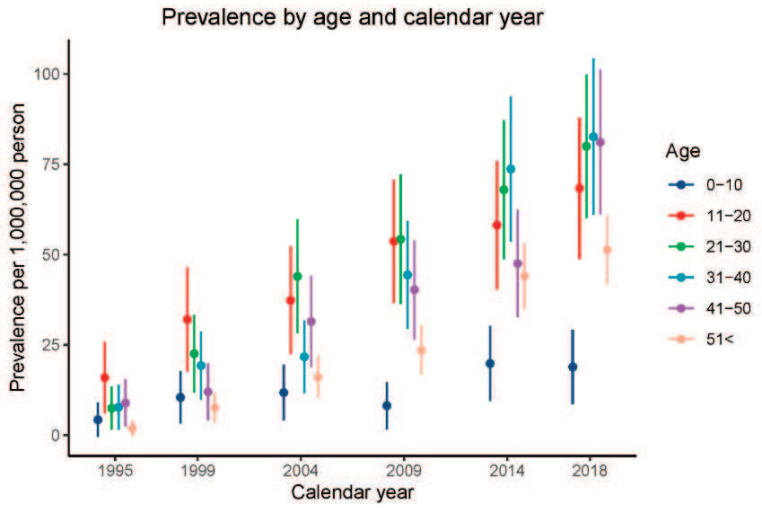

Figure 5. Prevalence by age and calendar year

Table 5. Prevalence by age and sex in 2018

Age	Men			Women		
	Prevalence per 1,000,000 person	Lower CI (95%)	Upper CI (95%)	Prevalence per 1,000,000 person	Lower CI (95%)	Upper CI (95%)
0-10	19.8	5.1	34.4	17.9	3.6	32.2
11-20	73.8	45.4	102.2	62.6	35.8	89.4
21-30	73.3	46.6	100.0	87.0	57.3	116.6
31-40	81.6	51.4	111.8	83.7	52.7	114.7
41-50	76.9	49.4	104.5	85.4	56.3	114.5
51<	39.2	27.2	51.2	62.5	48.0	77.1

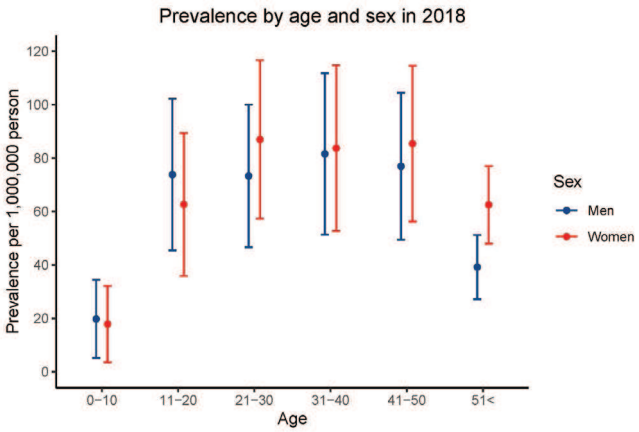

Figure 6. Prevalence by age and sex in 2018

Hypophosphatemia

No FD/MAS patients were coded with an ICD-10 code for hypophosphatemia or other disorders of mineral metabolism in the Danish National Patient Registry. No prescriptions were identified in the registers for vitamin D analogues or phosphate supplementation in FD/MAS. Therefore, the incidence and prevalence of hypophosphatemia in patients with FD/MAS could not be calculated.

Differentiation between PFD and MAS

Of the total of 408 patients, 29 (7.1%) were diagnosed with at least one of the ICD-10 codes of Table A, 9 (2.2%) were prescribed at least one of the drugs, and 35 (8.6%) had at least one encounter of either an ICD-10 code or one of the drugs. Several extraskeletal manifestations were diagnosed in 5 or less patients. The number of patients with a diagnosis for precocious puberty or with puberty-delaying therapy was 11 (2.6%). For pituitary hyperfunctioning, the number of diagnosed patients was 9 (2.2%), with no records of treatment. Hyperthyroidism was coded in 15 patients (3.6%) without records of treatment, hypercortisolism in <=5 patients with merely ketoconazole recorded in <=5 patients, hyperparathyroidism was diagnosed in <=5 patients, without records of treatment, and café au lait spots were coded in <=5 patients.

Discussion

This study provides insight in the incidence and prevalence of FD/MAS in Denmark. In recent years, the overall incidence of FD/MAS was 3.6 [95%CI: 2.9,4.5] per 1,000,000 person-years, meeting the definition of the EU for a rare disease[15]. The observed prevalence of FD/MAS was 61.0 [95%CI: 54.6,67.4]

per 1,000,000 persons, equivalent to 1 case per 16,500 [95% CI: 14.837,18.315] persons. Previous papers estimated FD to account for 5-7% of all benign bone tumors[16, 17], although these numbers were not based on scientific data. Research on FD/MAS in general is mostly conducted in specialized academic hospitals, where bias is imminent due to inclusion of mainly severely affected cases, which prevents accurate epidemiological research. Registry-based studies are less affected by referral or selection bias, and our cohort study is the first to investigate incidence and prevalence of FD/MAS in a nation-wide setting. In the Danish health care system, hospital care is free and universal, limiting selection bias regarding income, health insurance systems, age or hospital-specific cohorts[13]. The Danish National Patient Registry has a nation-wide coverage and data are prospectively collected. In general, the validity of ICD-10 coding in this registry is high, demonstrated by high completeness and high positive predictive value[11], although no studies have investigated these measures for FD/MAS specifically. However, a limitation of our study is that the prevalence calculations were affected by the revision of the ICD-8 to ICD-10 version in 1994, prior to the start of this study in 1995. The ICD revision caused left-truncation and brought a decreased prevalence of FD/MAS in older age groups: patients diagnosed with FD/MAS in the ICD-8 coding system, not repeatedly coded in ICD-10, were missed in our study. Specifically older patients with FD/MAS have lived more life years before 1994 than younger patients, leading to a higher chance to be diagnosed with FD/MAS before 1994 in the ICD-8 coding system and not (again) after 1994 in the ICD-10 coding system. Thus, the older patients are, the higher the chance on missing an ICD-10 diagnosis, and the larger the underestimation (figure 7). This phenomenon was merely absent in the age category 0-10 after 2004, as these patients were born after the revision, which therefore reflects the true prevalence (figure 5). Additionally, patients with merely craniofacial FD are missed. Since a very high number of cases was diagnosed with this code, we suspected a considerable dilution of this subgroup with patients with other fibrous jaw lesions and therefore a low positive predictive value of this code. Weighing consequences of over- and underdiagnosis, we excluded patients with this code. This problem in registry-based studies can only be fixed by creating a diagnosis code specifically for craniofacial fibrous dysplasia. Further analyses would also benefit from assigning separate codes to polyostotic disease as well as to McCune-Albright syndrome. In a similar manner, asymptomatic non-diagnosed cases are likely to be missed, a limitation hardly possible to address in any kind of research regarding FD/MAS. Despite these limitations, the epidemiology measures resulting from this study do provide understanding on the distribution of clinical apparent FD/MAS and are useful for various purposes. This may aid in screening and counseling of patients and may improve planning and budgeting of health care. Notably, consequences for planning and budget-

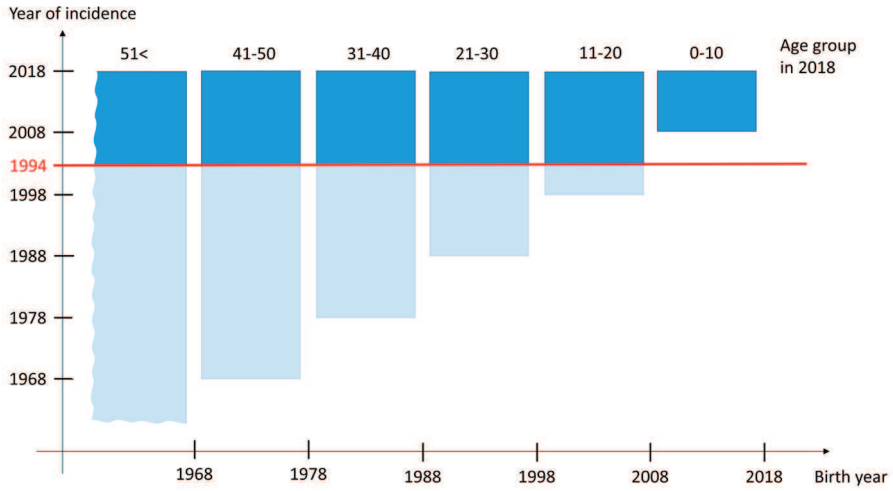

Figure 7. Year of incidence by birth year for all age groups

The blue area indicates the birth year and year of incidence range for each age group separately. E.g. those FD/MAS cases belong to age group 11-20 in 2018, who were born between 1998-2008 and were incident between 1998-2018. The total blue bar represents the total of the patients with FD/MAS in the age group. The red line marks the year 1994, where the ICD coding changed from the 9th to the 10th revision. The dark blue component of the bar represents the patients actually diagnosed in the ICD-10 coding system and included in this study. The light blue component represents the patients with FD/MAS coded by ICD-9 before 1994. These patients are missed in the current study if they were not reportedly diagnosed in the ICD-10 system. The chart reflects that in older age groups, higher ratio of cases are missed in this study, i.e. the underestimation is higher for older age groups.

ing health care are not affected by the missingness of asymptomatic cases, as these patients do not require health care. Our results also allow comparison with other rare bone diseases/skeletal dysplasias. The incidence and prevalence of FD/MAS are low compared to osteogenesis imperfecta (OI) in Denmark (incidence 150 per 1,000,000 births, birth and population prevalence 218 and 106 respectively per 1,000,000 persons[18, 19]. Incidence is vastly lower than in X-linked hypophosphatemic rickets (XLH) (39 per 1,000,000 children of age 0-15 years) although the prevalence is similar (48 per 1,000,000 children)[20]. Prevalence of FD/MAS is similar to birth prevalence of achondroplasia (46 per 1,000,000)[21] but lower than prevalence of hypophosphatasia (157 per 1,000,000)[22]. Although most cases of OI, XLH and achondroplasia are diagnosed at birth, prevalence in FD/MAS may not be directly comparable to birth or childhood prevalence due to methodological differences. The incidence of FD/MAS is a fraction compared to OI and XLH, but the prevalence is similar to XLH and achondroplasia and approximately half of OI and hypophosphatasia. This is probably since FD/MAS is

diagnosed in a broad age range, while other skeletal dysplasias are diagnosed in early childhood. Lastly, future studies may benefit from results of this study: the impact of future therapeutic targets or other interventions may be estimated according to the prevalence of FD/MAS reported in this study. Prevalence monotonously increased from one calendar year to the next, as expected for a chronic disease that is not believed to materially affect life expectancy, with increasing incidence rates.

Apart from incidence and prevalence in general, our study provided insight in temporal change. The incidence rate of FD/MAS was higher in the second half of the study compared to the first half, with a rise in incidence around 2007 and a stable rate in the last 10 years. This trend may be due to growing awareness, intensified screening protocols, improvements in technical abilities or detection methods, or increased usage of radiology for other conditions. We had expected these factors to diminish diagnostic delay and result in diagnosis at younger age in later calendar intervals, but surprisingly this is not supported by our data: in all calendar years, the highest incidence was observed in age group 11-20 years, followed by 0-10 years, with consistently lower rates in adulthood. Probably children and teenagers do not receive radiographic assessment in absence of (severe) complaints. Yet small decreases in diagnostic lag time could have been missed due to our 10-year age intervals. The age-specific pattern of diagnosis is consistent with previous studies, demonstrating most disease progression[21, 22] and fractures[23] in FD/MAS in the first 2 decades of life. The rise in incidence is mainly due to an increased rate of monostotic FD diagnoses in last 10 years, as incidence of polyostotic FD/McCune-Albright syndrome was stable over time. Specifically for monostotic FD, innovations in imaging and histologic techniques, including GNAS mutation screening, may have augmented differentiation between a monostotic FD lesion and other bone lesions, while the diagnosis of PFD/MAS is less dependent on radiology and pathology, due to the more severe phenotype of multiple lesions and shorter list of possible differential diagnoses. Additionally, incidental diagnosis of clinically silent MFD is presumably enhanced over time by the broader use and improved quality of radiographic imaging for musculoskeletal complaints in general, which does not apply for the mainly symptomatic subtypes PFD and MAS. In the final years of the study, 66% of patients were diagnosed with monostotic FD compared to 34% with polyostotic FD/McCune-Albright syndrome. Previous studies have reported McCune-Albright syndrome to represent 7-80% of cases[23-25], heavily dependent on the type of hospital and degree of bias. In our study, the identification of patients with MAS was attempted by evaluation of diagnoses codes for extraskeletal manifestations of MAS and drug codes for their treatment. This analyses demonstrated 8.6% of patients diagnosed with or treated for extraskeletal disease,

which is at the lower range of the proportion found in literature. Unfortunately, these analyses were of limited value due to many categories with <=5 cases and due to several limitations. To capture as many cases as possible, a wide range of possible ICD-10 codes was selected, yet underdiagnosing and underreporting might be a confounding issue. Other limitations include absence of data on endocrine disease frequency in the general Danish population, preventing formal comparison, and the absence of clinical data to confirm these endocrinopathies to be MAS-related and confirm these drugs to be prescribed for MAS-related endocrinopathies. For these reasons, this study merely provides valuable data on the epidemiological differences between MFD and PFD/MAS, which may benefit assessment of bias in cohort studies. An important difference between MFD and PFD/MAS subgroups was age of diagnosis: cases diagnosed at older age were mostly patients with monostotic disease, with a proportion certainly comprising incidental findings in asymptomatic patients. Polyostotic FD/McCune-Albright syndrome was less common, but more frequently diagnosed in the first decade of life due to the more severe phenotype. Therefore it should be considered that the majority of patients will not develop the severe consequences of FD/MAS reported in some studies conducted in a severely affected population of FD/MAS. Especially when patients are diagnosed at older age, the risk for a severe phenotype and associated osteomalacia, progressive deformity, nerve compression or fractures diminishes[24, 26].

Although also set up to provide an answer on the incidence of hypophosphatemia, no patients were coded with an ICD-10 code for hypophosphatemia nor were identified via prescriptions for vitamin D analogues or phosphate supplementation. This is contrary to literature cohorts[4, 7, 26, 27] and we would have expected a match in at least the PFD group. This can reasonably be explained by the manner of registration or prescription coding, as it is not mandatory to register secondary diagnoses in Denmark, but might also be due to underdiagnosis and undertreatment of hypophosphatemia in persons with FD/MAS. Biochemistry data could be useful to identify cases of FD/MAS with hypophosphatemia and to determinate the underlying mechanism for the lack of registration, but these were not available for this study.

In conclusion, the incidence rate of FD/MAS in Denmark was 3.6 [95%CI: 2.9,4.5] per 1,000,000 person-years and the prevalence was 61.0 [95%CI: 54.6,67.4] per 1,000,000 persons. Most diagnoses were established between the age of 11 and 20 years, although MFD was more often diagnosed at older age than PFD/MAS. Two-thirds of the FD/MAS population was diagnosed with MFD, one-third with PFD/MAS. These epidemiology measures provide understanding on the distri-

bution of disease, may aid in screening for FD/MAS, in planning health care, and in counselling patients, and may benefit future research.

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Supplementary files

Table A. ICD-10 codes and ATC-codes to identify MAS-related extraskeletal manifestations with number of patients in study population

Extraskeletal manifestation	ICD-10 code	Title of code	ATC code	Title of drug	Total N of patients (%)
Precocious puberty	E30.1 E30.8 E30.9 E28.0 E28.1	Precocious puberty Other disorders of puberty Disorder of puberty, unspecified Estrogen excess Androgen excess	L02BG L02BA L02AE	Aromatase inhibitors Anti-estrogens GnRH analogues	11 (2.6%)
Hyperpituitarism	E22.0 E22.1 E22.8 E22.9	Acromegaly and pituitary gigantism Hyperprolactinaemia Other hyperfunction of pituitary gland Hyperfunction of pituitary gland, unspecified	H01CB H01AX	Somatostatin and analogues Other pituitary hormones/ analogues	9 (2.2%)
Hyperthyroidism	E05.0 E05.1 E05.2 E05.3 E05.4 E05.8 E05.9	Thyrotoxicosis with diffuse goiter Thyrotoxicosis with toxic single thyroid Thyrotoxicosis with toxic multinodular goiter Thyrotoxicosis from ectopic thyroid tissue Thyrotoxicosis factitial Other thyrotoxicosis Thyrotoxicosis, unspecified	H03BB	Sulfur-containing imidazole derivatives	15 (3.6%)
Neonatal Cushing/ hypercortisolism	E24.8 E24.9 E27.8 E27.9	Other Cushing's syndrome Cushing's syndrome, unspecified Other specified disorders of adrenal gland Disorder of adrenal gland, unspecified	V04CD01 N01AX07 L01XX23 J02AB02 H02CA	Metyrapone Etomidate Mitotane Ketoconazole Anticorticosteroids	<=5
Primary hyperparathyroidism	E21.0 E21.3 E21.4 E21.5	Primary hyperparathyroidism Hyperparathyroidism, unspecified Other specified disorders of parathyroid gland Disorder of parathyroid gland, unspecified	H05BX01	Cinacalcet	<=5
Café au lait	L81.3	Café au lait spots	NA	NA	<=5
Other/general	E35*	Disorders of endocrine glands in diseases classified elsewhere	NA	NA	<=5
Total	-	-	-	-	35 (8.6%)

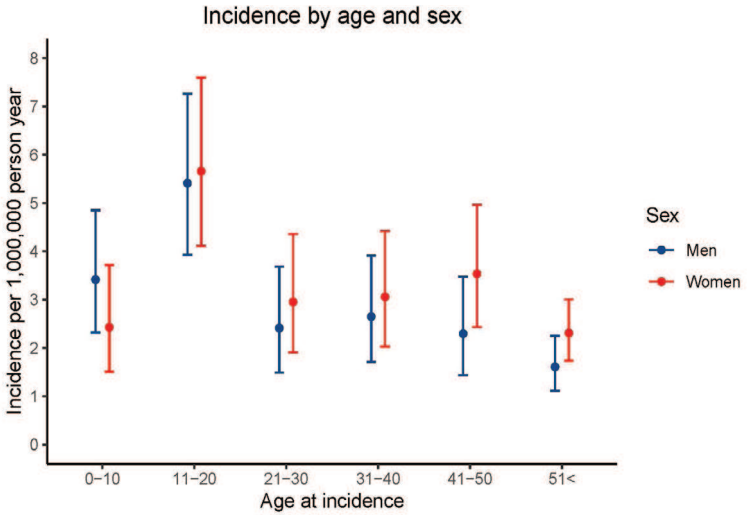

Figure A. Incidence by age and sex

Table B. Incidence by age and sex

Age at incidence	Men			Women		
	Incidence rate per 1,000,000 person year	Lower CI (95%)	Upper CI (95%)	Incidence rate per 1,000,000 person year	Lower CI (95%)	Upper CI (95%)
0-10	3.42	2.32	4.85	2.43	1.50	3.72
11-20	5.41	3.93	7.26	5.66	4.11	7.60
21-30	2.41	1.49	3.69	2.95	1.91	4.36
31-40	2.65	1.71	3.91	3.06	2.03	4.42
41-50	2.30	1.44	3.48	3.53	2.43	4.96
51<	1.61	1.11	2.25	2.31	1.74	3.00

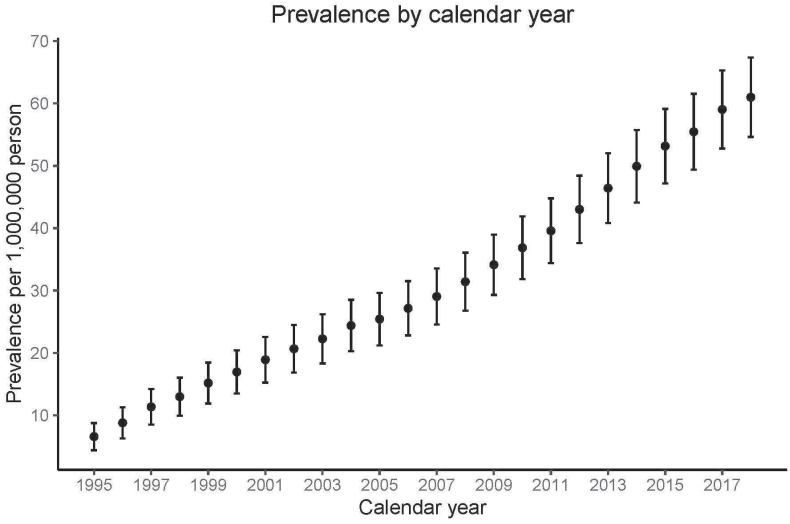

Figure B. Prevalence by calendar year

Table C. Prevalence by age and diagnosis code in 2018

Age	M85.0			Q78.1		
	Prevalence per 1,000,000 person	**Lower CI (95%)**	**Upper CI (95%)**	**Prevalence per 1,000,000 person**	**Lower CI (95%)**	**Upper CI (95%)**
0-10	11.6	3.6	0-10	11.6	3.6	0-10
11-20	42.2	26.8	11-20	42.2	26.8	11-20
21-30	46.4	31.3	21-30	46.4	31.3	21-30
31-40	51.6	34.5	31-40	51.6	34.5	31-40
41-50	60.5	43.2	41-50	60.5	43.2	41-50
51<	36.2	28.2	51<	36.2	28.2	51<

Chapter 6

Coxa vara deformity in Fibrous dysplasia/ McCune-Albright syndrome: prevalence, natural history and risk factors, a two-center study

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Abstract

This study aimed to evaluate the prevalence of and risk factors for coxa vara deformity in patients with Fibrous Dysplasia/McCune-Albright Syndrome (FD/MAS). This study was conducted at the National Institutes of Health and Leiden University Medical Center. All patients with any subtype of FD/MAS, FD involving the proximal femur, ≥1 X-ray available and age <30 years were included. X-rays were scored for the neck-shaft angle (NSA). Varus deformity was defined as NSA <110° or >10° below age-specific values. Risk factors for deformity were assessed by nested case-control analysis, comparing patients and femurs with and without deformity, and by linear mixed effects model, modelling temporal NSA decrease (the natural course of the NSA) in non-operated femurs with ≥2 X-rays. Assessed variables included growth hormone excess, hyperthyroidism, hypophosphatemia, >25% of the femur affected, calcar destruction, radiolucency and bilateral involvement. In total 180 patients were studied, 57% female. Mean baseline age was 13.6 (±SD 7.5) years; median follow-up 5.4 (IQR 11.1) years. 63% were diagnosed with MAS. 94 patients were affected bilaterally; 274 FD-femurs were analyzed; 99 femurs had a varus deformity (36%). In the nested case-control analysis, risk factors were: presence of MAS ($p<0.001$), hyperthyroidism ($p<0.001$), hypophosphatemia ($p<0.001$), high percentage of femur affected ($p<0.001$), calcar destruction ($p<0.001$). The linear mixed effects model included 114 femurs, identified risk factors were: growth hormone excess ($\beta=7.2$, $p=0.013$), hyperthyroidism ($\beta=11.3$, $p<0.001$), >25% of the femur affected ($\beta=13.2$, $p=0.046$), calcar destruction ($\beta=8.3$, $p=0.004$), radiolucency ($\beta=3.9$, $p=0.009$), bilateral involvement ($\beta=9.8$, $p=0.010$). Visual inspection of the graph of the model demonstrated most progression of deformity if NSA <120° with age <15. In conclusion, in tertiary care centers, the prevalence of FD/MAS coxa vara deformity was 36%. Risk factors included presence of MAS, high percentage of femur affected, calcar destruction, radiolucency, NSA <120° and age <15.

Introduction

In fibrous dysplasia/McCune-Albright syndrome (FD/MAS), postzygotic variants in *GNAS* induce abnormalities in skeletal, endocrine and dermal tissue[1]. Fibrous, woven bone lesions may be localized in one skeletal site (monostotic FD, MFD) or several bones (polyostotic FD, PFD). MAS is diagnosed in patients with ≥2 (extra)skeletal features[2]. The official term for the disorder is FD/MAS and this includes the entire spectrum of disease with all subtypes of FD/MAS. The affected proximal femur is especially susceptible to deformation, given the weight-bearing forces and vulnerability to (micro)fractures. Coxa vara and lateral bowing may occur and progress during childhood and adolescence to the Shepherd's crook deformity with secondary pain, (micro)fractures and disability[3, 4]. Factors proposed to be associated with deformity include young age at diagnosis, MAS (specifically hyperthyroidism and FGF23-mediated hypophosphatemia with reduced mineralization), increased serum alkaline phosphatase, fractures, extensive metaphyseal lesions, cortical thinning, cystic lesions eroding the calcar femorale and weightbearing[3, 5]. However, these studies had several limitations, investigating a limited number of risk factors or patients, or establishing risk factors in patients with deformity without statistical comparison to non-deformed patients.

In children with deformity, early surgery may be necessary to prevent progression, but difficulties encompass peri-implant fracture; hardware failure in weakened bone without normal bone for bridging; implantation difficulties due to severe deformity; standard implants not fitting in pediatric bone vs. pediatric implants not preventing deformity; growth plate preservation; and recurrence requiring multiple surgeries. Data on the optimal timing and techniques are scarce [6-9]. These surgical difficulties underline the need for strategies to prevent or decelerate progression of deformity.

To optimize treatment of children with FD in the proximal femur, it is necessary to assess the prevalence of deformity and to explore which patients are at risk for deformity. Aims of this study are to establish the prevalence and natural course of coxa vara deformity, and risk factors for development and progression in children, adolescents and young adults with FD/MAS in two expert centers.

Methods

Population

This multicenter cohort study was conducted at the Leiden University Medical Center (LUMC) and National Institutes of Health (NIH), both tertiary expert centers for FD/MAS. Inclusion criteria were: age <30 years upon hospital intake, confirmed diagnosis of any FD/MAS subtype (monostotic fibrous dysplasia, polyostotic fibrous dysplasia or McCune-Albright syndrome), FD involving the metaphysis or proximal epiphysis of at least one femur and ≥1 available X-ray. Eighty-five patients were excluded due to age >30 years upon intake, 12 patients excluded with lesional tissue merely in the femoral shaft or distal femur, and 25 patients excluded due to imaging being unavailable or of insufficient quality. Patients at LUMC were followed until end of hospital follow-up. Patients at NIH were seen as part of a natural history study approved by the Investigational Review Board. The Medical Ethics Committee of LUMC approved the study. Patients/guardians provided informed consent/assent for inclusion. Several included patients were also included in previous studies from both centers[10-12], but data were not re-used and were collected and analyzed independently for this present paper.

Parameters

Clinical characteristics

Patients' characteristics were retrieved from health records and included sex, age, hospital, FD/MAS subtype (monostotic disease or polyostotic disease or McCune-Albright syndrome), history of extraskeletal manifestations, laboratory values, body mass index, presence and severity of pain, mobility (classified as wheelchair user, assisted with walking device, unassisted full weight bearing with sports restrictions, or unassisted full weight bearing without restrictions), history of treatment with phosphate/vitamin D/calcium supplementation, bisphosphonates, denosumab or therapy for endocrinopathies.

Radiographical examination

The first available radiograph (X-ray or CT, but not MRI) of all affected femurs was identified as baseline radiograph and scored for the following items. The location of the lesion within the proximal femur was assessed and scored as presence or absence of involvement of the femoral head, neck, area near the greater trochanter, area near the lesser trochanter or intertrochanteric area. Lesion extent was measured by estimating the percentage of affected tissue on the total femoral area, categorized as 0-25%, 25-50%, 50-75 or >75%. Involvement of the femoral calcar with FD was defined as present or absent. The smallest cortical thickness of the femoral neck was measured and categorized as 0-1 mm, 1-2

mm and >2 mm. Destruction of the femoral calcar was scored as present if this cortical thickness was smaller than the healthy contralateral side, below 2 mm, or if the calcar was visually irregular or indented. Cystic appearance was scored as present in case of radiolucency in comparison to surrounding normal bone. The presence of (stress)fractures was assessed and the presence of diaphyseal deformity. Lastly, the neck-shaft angle (NSA) was measured, but only in absence of internal/external rotation or if rotation was consistent over multiple X-rays. To evaluate the natural course of femoral varisation and deformation, all patients were identified with ≥2 radiographs of the affected, non-operated femur or ≥2 radiographs of the affected operated femur prior to surgery. All described assessments were repeated on all radiographs of these non-operated femurs until end of follow-up (i.e. age 30 years or surgery, whichever occurred first). The NSA measurement has excellent intra- and interclass correlation FD[10].

Classification of deformity

The NSA of the last radiograph was used to evaluate whether femurs developed deformity. This study focused on varus deformity, diaphyseal deformity was not considered. For our study, varus deformity was defined as NSA <110° (based on the general definition of coxa vara[13, 14]) or >10° below the healthy contralateral side or age-specific values (according to Tachdjian[15] and measurement reliability in literature[16-18]). Patients were also classified into deformity types according to the scoring system of Ippolito et al, which is based on deformity in the diaphysis (absent in type 1-3, present in type 4-6) and on deformity in the proximal femur (absent in type 1 and 4, valgus deformity in type 2 and 5, varus deformity in type 3 and 6). Type 0 indicates no deformity, type 1 solely irregular cortical surfaces[19].

Analyses

Statistical analyses were performed in SPSS Statistics v25 (IBM Corp., Armonk, NY, USA). Baseline characteristics were summarized by descriptive statistics. Mean (± standard deviation) was used for normally distributed continuous variables, otherwise median (quartile 1-3). The association between lesional size and diagnosis of McCune-Albright syndrome and was evaluated by comparing the median of the estimated femoral area involved by FD between patients with and without McCune-Albright syndrome by Wilcoxon signed-rank (e.g. if 25-50% of the femoral area is affected by FD in a patient, the median percentage used for the patient for this comparison was 37.5%). P-values <0.05 were considered statistically significant. The prevalence of varus deformity was calculated by dividing the number of femurs developing deformity during follow-up by the total number of affected femurs.

Risk factors

Two methods were used to evaluate risk factors for deformity: a nested case-control analysis (cross-sectional) and statistical modelling of NSA by age (longitudinal). The case-control analysis included all femurs regardless of treatment history. For patient-specific risk factors (sex, age intake, subtype of FD/MAS, presence of extraskeletal manifestations), patients with bilateral or unilateral deformity at the end of follow-up (cases) were compared with patients without deformity (controls) by one-way ANOVA and chi-squared test with pairwise comparisons. For femur-specific risk factors (lesion location, percentage of femoral area affected by FD, radiolucent appearance, involvement/destruction of femoral calcar), deformed femurs (cases) were compared with non-deformed femurs (controls) by independent t-test or chi-squared test. P-values <0.05 were considered statistically significant. The second approach was a linear mixed effects model to analyze the natural course of the NSA over time. This was conducted in the subgroup of patients with ≥2 radiographs of the affected non-operated femur or pre-surgery, in which radiographic assessments were repeated over time. In this model, each femur represented a case (n=114) and a maximum of 4 repeated measurements per case was included. The assumption of a normal distribution for the dependent variable NSA was met. The assumption of the missing data mechanism being at random was also confirmed: the number of measurements was comparable between patients with or without McCune-Albright syndrome and with or without deformity, demonstrating no association between disease severity and loss to follow-up. Missing data were not imputed, as the model accounts for missing data. Time was expressed as the age of the patient at the time of the X-ray. Age was used as continuous variable, and an additional quadratic effect of age was added, as this significantly improved the fit of the model. Hypothesized risk factors, based on literature and the initial results of the case-control analysis, were entered as fixed factors in the model and included history of growth hormone excess, hyperthyroidism or hypophosphatemia, increased bone turnover markers, >25% of femoral area involved by FD, calcar destruction, radiolucency and bilateral involvement. Additionally, the hospital of inclusion was entered as fixed factor to determine between-hospital differences. It was hypothesized that an interaction was present between age and calcar destruction and age with >25% of the femoral area affected, therefore these interactions terms were entered as fixed factors. A random intercept was included and the covariance matrix was scaled identity. Results are reported as regression coefficient β, with confidence interval (CI) and p-value. P-values <0.05 were considered statistically significant.

Results

Baseline characteristics

A total of 180 patients were included, the majority was included from NIH (n=107, 60%), female (n=102, 57%) and diagnosed with McCune-Albright syndrome (n=113, 63%). In patients with McCune-Albright syndrome, the most common extra skeletal manifestation was hyperpigmented macules (57%) and precocious puberty (46%). Baseline characteristics are summarized in table 1. Ninety-four patients were bilaterally affected with fibrous dysplasia (52%), therefore in total 274 femurs were evaluated. Lesion characteristics are summarized in table 2. Patients with McCune-Albright syndrome had larger lesions than patients with FD without extraskeletal features (respectively median 87.5% of femoral area involved by FD vs 15%, p<0.001). Ninety-nine femurs had developed varus deformity at the end of follow-up, providing a prevalence of 36.1%. Radiolucency and calcar destruction were frequently present at baseline, on 50% and 69% of X-rays respectively.

Table 1. Baseline characteristics

Number of patients per site	**LUMC (N=73, 40.5%)**	**NIH (N=107, 59.4%)**	**Total (N=180)**
Sex, female (n, %)	37 (50.7%)	65 (60.7%)	102 (56.7%)
Age, years (mean ±SD)			
At inclusion (intake hospital)	18 (±6.7)	10.6 (±6.4)	13.6 (±7.5)
At end FU (last hospital visit)	28.4 (±11.8)	16.7 (±8.9)	21.5 (±11.7)
Type of FD (n, %)			
Monostotic	37 (50.7%)	1 (0.9%)	38 (21.1%)
Polyostotic	23 (31.5%)	6 (5.6%)	29 (16.1%)
McCune-Albright syndrome	13 (17.8%)	100 (93.5%)	113 (62.8%)
Extraskeletal manifestations (n, %)			
Precocious puberty	11 (17.7%)	67 (62.6%)	78 (46.2%)
Growth hormone excess	7 (9.6%)	29 (27.1)	36 (20.0%)
Hyperprolactinemia	5 (6.8%)	19 (17.8%)	24 (13.3%)
Hyperthyroidism	5 (6.8%)	47 (43.9%)	52 (28.9%)
FGF-23 m. hypophosphatemia	9 (15.0%)	40 (37.4%)	49 (29.3%)
Hyperpigmented macules	12 (16.4%)	90 (84.1%)	102 (56.7%)
Affected femur (n, %)			
Right	24 (32.9%)	14 (13.1%)	38 (21.1%)
Left	34 (46.6%)	24 (13.1%)	48 (26.7%)
Both	15 (20.5%)	79 (73.8%)	94 (52.2%)
Pain present at baseline (n, %)	55 (75.3%)	49 (45.8%)	104 (57.8%)
Mobility at baseline (n, %)			
Unassisted, no sports restrictions	29 (39.7%)	51 (47.7%)	80 (44.4%)
Unassisted with sports restrictions	23 (31.5%)	13 (12.1%)	36 (20.0%)
Assisted with walking device	7 (9.6%)	26 (24.3%)	33 (18.3%)
Wheelchair user	9 (12.3%)	10 (9.3%)	19 (10.6%)
BMI > 25 at baseline (n, %)	10 (13.7%)	19 (17.8%)	29 (16.1%)

Table 2. Lesion characteristics

Total number of femurs	**N=274**
Lesion location (n, %)	
Head	134 (48.9%)
Neck	258 (94.2%)
Near greater trochanter	223 (81.4%)
Near lesser trochanter	264 (96.4%)
Intertrochanteric	261 (95.3%)
Entire proximal femur	122 (44.5%)
Extent (n, %)	
Unifocal	66 (24.1%)
Multifocal	208 (75.9%)
Amount of femur affected (n, %)	
0-25%	54 (19.7%)
25-50%	30 (10.9%)
50-75%	51 (18.6%)
>75%	139 (50.7%)
Deformity type (Ippolito) end follow-up (n, %)	
0	32 (11.7%)
1	65 (23.7%)
2	2 (0.7%)
3	15 (5.4%)
4	65 (23.7%)
5	2 (0.7%)
6	84 (30.7%)
Deformity (n, %)	
Development of varus deformity	99 (36.1%)
Development of subtrochanteric deformity	157 (57.3%)
Radiolucency at baseline (n, %)	137 (50.0%)
Calcar involvement at baseline (n, %)	244 (89.1%)
Calcar destruction at baseline (n, %)	188 (68.6%)
Smallest calcar thickness at baseline	
0-1 mm	123 (44.9%)
1-2 mm	55 (20.1%)
> 2 mm	46 (16.8%)
Not measurable	50 (18.2%)

Case-control analysis

Patients without deformity were older at hospital intake than patients with bilateral deformity (table 3) (14.1 ±7.8 vs 7.4 ±6.7 years respectively, p=0.004). The proportion of patients with McCune-Albright syndrome and specifically with growth precocious puberty, growth hormone excess, hyperthyroidism and hypophosphatemia increased from the group without deformity to the group with unilateral deformity and bilateral deformity. Femurs developing deformity had larger lesions compared to non-deformed femurs: the entire proximal femur was involved in 70.7% of deformed vs 28.7% of non-deformed femurs (p<0.001), and the percentage of femoral area affected was larger in deformed than

non-deformed femurs (0<0.001) (table 4). Calcar destruction, additional shaft deformity, decreased leg length, fractures and surgeries were more common in deformed femurs compared to non-deformed femurs (table 4).

Linear mixed effects model

The mean NSA was 138.5, 95%CI [130.5,146.4] at baseline. The NSA decreased significantly over time (i.e. when patients grow older), with a linear and quadratic effect of the time variable age (table 5, figure 1). Femurs developing deformity demonstrated most progression before age 15 and below angle 120° (figure 1). Risk factors for a decrease in NSA were as follows. Calcar destruction contributed to a lower NSA as main effect (β= -8.3, 95%CI[-13.9,-2.6], p=0.004) as well as in the interaction with age (β= -0.8, 95%CI[-1.2,-0.4], p<0.001), indicating that the NSA was on average 8.3, 95%CI[-13.9,-2.6] degrees lower and demonstrated a steeper decline over time in patients with calcar destruction compared to patients without. This was similar for the percentage of femoral area involved by FD: patients with more than 25% of the femoral area affected had a 13.2, 95%CI[-0.2,-26.2] degrees lower NSA compared to patients with less than 25% of the femoral area affected (β= -13.2, 95%CI[-0.2,-26.2], p=0.046), and had additionally a steeper decline over time (interaction of >25% of femoral area affected with age: β= -1.2, 95%CI[-1.8,-0.5], p=0.001). Furthermore, risk factors for a lower NSA included radiolucent appearance, providing a change in NSA of -3.9, 95%CI[-6.9,-1.0] degrees if present, bilateral involvement (NSA -9.8, 95%CI[-17.2,-2.4]), growth hormone excess (NSA -7.2, 95%CI[-12.8,-1.5] degrees) and hyperthyroidism (NSA -11.3, 95%CI[-16.9,-5.6]).

Table 3. Comparison of characteristics in patients with and without deformity

Number of patients per group	**Bilateral deformity (n=30)**	**Unilateral deformity (n=39)**	**No deformity (n=109)**	**p-value**
Sex, female (n, %)	18 (60.0%)	22 (56.4%)	61 (56.0%)	p=0.978
Age intake, years (mean ±SD)	7.4 (6.7) a	12.7 (6.4) a,b	14.1 (7.8) b	p=0.004
Type of FD (n, %)				
Monostotic	0	3 (7.7%)	34 (31.2%)	p<0.001
Polyostotic	0	6 (15.4%)	23 (21.1%)	
McCune-Albright syndrome	30 (100%) a	30 (76.9%) b	52 (47.7%) c	
Extraskeletal manifestations (n, %)				
Precocious puberty	23 (76.7%) a	19 (48.7%) a,b	35 (32.1%) b	p=0.001
Growth hormone excess	10 (33.3%)	11 (28.2%)	15 (13.8%)	p=0.085
Hyperprolactinemia	7 (23.3%)	6 (15.4%)	10 (9.2%)	p=0.132
Hyperthyroidism	21 (70%) a	14 (35.9%) b	17 (15.6%) c	p<0.001
FGF-23 m. hypophosphosphatemia	19 (63.3%) a	13 (33.3%) a,b	16 (14.7%) b	p<0.001
BMI >25 during follow-up (n, %)	10 (33.3%)	7 (17.9%)	27 (24.8%)	p=0.060
Pain during follow-up (n, %)	21 (70%)	28 (71.8%)	84 (77.1%)	p=0.068

One-way ANOVA for comparing continuous outcomes, chi square for comparing categorical outcomes.

Letters a, b, and c refer to the difference in subgroups. Subgroups with similar letters are not statistically different, groups with different letters are.

Definition of deformity: NSA <110° or >10° below healthy contralateral side or age-specific values.

NB: patients with unilateral deformity include patients with 1 femur affected and deformed, or patients with 2 femurs affected, but merely 1 deformed.

Table 4. Comparison of characteristics in femurs with and without varus deformity

Number of femurs	**Development of varus deformity (n=99)**	**No development of varus deformity (n=167)**	**p-value**
Lesion location (n, %)			
Head	72 (72.7%)	61 (36.5%)	p<0.001
Neck	98 (99.0%)	153 (91.6%)	
Near greater trochanter	90 (90.9%)	119 (71.3%)	
Near lesser trochanter	99 (100%)	159 (95.2%)	
Intertrochanteric	99 (100%)	153 (91.7%)	
Entire proximal femur	70 (70.7%)	48 (28.7%)	
Area of femur affected (n, %)			
0-25%	6 (6.0%)	46 (27.5%)	p<0.001
25-50%	8 (8.1%)	27 (16.2%)	
50-75%	14 (14.1%)	36 (21.6%)	
>75%	71 (71.7%)	58 (34.7%)	
Radiolucency at baseline	53 (53.5%)	80 (47.9%)	p=0.375
Involvement of calcar at baseline	97 (98.0%)	135 (80.8%)	p<0.001
Calcar destruction at baseline	83 (83.8%)	92 (55.1%)	p<0.001
Smallest calcar thickness at baseline			
0-1 mm	64 (64.6%)	55 (32.9%)	
1-2 mm	12 (12.1%)	39 (23.4%)	
> 2 mm	7 (7.1%)	40 (24.0%)	
Not measurable	17 (17.2%)	33 (19.8%)	
Additional shaft deformity			
Yes	84 (84.8%)	68 (40.7%)	p<0.001
No	15 (15.2%)	99 (59.3%)	
Leg length difference compared to contralateral side (n, %)			p<0.001
Leg shorter	54 (54.5%)	47 (28.1%)	
Leg longer	28 (28.3%)	54 (32.3%)	
Leg equal	10 (10.1%)	49 (29.3%)	
Fractures during follow-up	40 (40.4%)	38 (22.8%)	p=0.002
No fractures during follow-up	59 (59.6%)	129 (77.2%)	
Surgery performed	78 (78.8%)	89 (53.3%)	p<0.001
Surgery not performed	21 (21.2%)	78 (46.7%)	

Independent t-test for comparing continuous outcomes, chi square for categorical outcomes.

Definition of deformity: NSA <110° or >10° below healthy contralateral side or age-specific values.

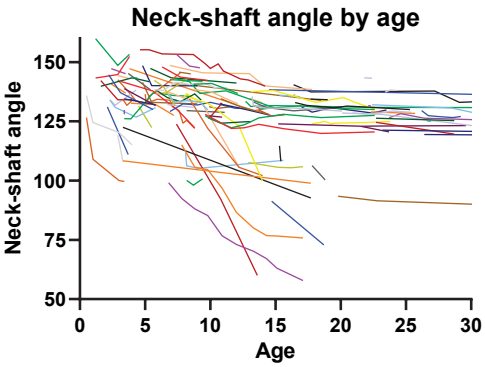

Figure 1. Neck-shaft angle by age

Natural progression of NSA by age, in patients with ≥2 available X-rays of the non-operated, affected femur. Each line represents a femur affected with FD. Follow-up ends if surgery is conducted. Deformity progression appears to occur before the age of 15, when the NSA declines below 120 degrees.

Discussion

Aims of this study were to evaluate the prevalence and natural course of and risk factors for coxa vara deformity in children, adolescents and young adults with FD/MAS. In our tertiary care center cohort, one-third of the affected femurs developed coxa vara. This prevalence is similar to previous studies by Ippolito (36%)[19] and Zhang (28%)[20], although the distribution of FD-subtypes was different in those studies and the cut-off value for coxa vara was 120°. In our study 110° was used, since this was more common in literature on coxa vara in general[13, 14, 21] and in other bone dysplasias[22-24].

Rapid progression of deformity was observed in children below the age of 15 years when the NSA declined below <120°. Increasing prevalence of deformity over time has been previously described, from 4% at diagnosis to 44% after 7 years[3], and age is a known indicator of disease progression and lesion expansion[4, 25]. Next to age, in our cohort a diagnosis of McCune-Albright syndrome was an important risk factor for deformity development and progression. Only patients with McCune-Albright syndrome demonstrated bilateral deformity, but of patients with unilateral deformity, 25% had no history of McCune-Albright syndrome and 8% was even diagnosed with monostotic fibrous dysplasia. This implies a lower but not absent risk for deformity in patients with monostotic or polyostotic disease without endocrine abnormalities, emphasizing the importance of monitoring, particularly in presence of other risk factors. We postulated that growth hormone excess and hyperthyroidism were risk factors in patients

with McCune-Albright syndrome, and FGF23-mediated hypophosphatemia in patients with severe skeletal involvement, as these features were proven to deteriorate bone quality in FD/MAS[11, 26, 27]. Indeed, both the case-control analysis and linear mixed effects model revealed growth hormone excess and hyperthyroidism as risk factors. Although hypophosphatemia occurred more frequently in patients with deformity, the linear mixed effects model did not identify it as risk factor. Possibly hypophosphatemia is associated with coxa vara development, but not directly related to temporal progression. Several studies demonstrated an association between increased bone turnover markers and deformity or lesion progression[5, 27, 28]. This was not apparent in our results, possibly because laboratory measurements were not systematically collected and mostly available in patients with severe disease and increased markers, reducing the discriminative use of this variable. In contrast, cystic appearance was a risk factor in the linear mixed effects model but not in the case-control analysis. The latter included radiographic features of all femurs at baseline regardless of treatment history, whereas the linear mixed effects model, modelling the natural course of varisation, included radiolucency on every X-ray of non-operated femurs. Temporal change in radiolucency, which was only seen in the linear mixed effects model, may explain the difference. Ippolito et al. likewise observed more osteolysis, calcar destruction and extensive lesional tissue in patients with progressive deformity[3]. Lastly, bilateral involvement posed as risk factor. Therefore, the risk profile for deformity development appears to be: children below age 15 with McCune-Albright syndrome, specifically growth hormone excess and hyperthyroidism, extensive skeletal involvement, calcar destruction, osteolysis, and declining angle below 120°. These findings lead us to propose yearly monitoring of these patients including assessment of the NSA, radiolucency, and calcar thickness; adequate screening for and treatment of endocrinopathies; and optimizing bone quality according to the guidelines[2]. Furthermore, we recommend to consider surgery when the NSA declines below 120°. The optimal time for surgery was only addressed in one expert opinion, recommending surgery below 120° [7]. Our results indeed provide data to support this, given the rapid decline below this threshold. Lastly, deformity was associated with leg shortening, fractures and surgery, but not presence of pain at intake.

Our study is the first to evaluate prevalence and etiology of coxa vara deformity in children and adolescents, to provide a guideline to identify patients at risk. The fact we investigated the largest cohort of patients with FD and femoral involvement to date, and used two methods for analyzing risk factors for deformity strengthens our results. However, limitations include the retrospective design and missing data on pain, mobility, and laboratory markers, which hampered the evaluation of any association between these factors and deformity. Secondly,

Table 5. Risk factors for progressive deformity (decrease in degrees of NSA)

Factor	Regression coefficient	95% CI	p-value
Intercept	138.5	130.5,146.4	<0.001
Age	-3.6	-4.2,-2.9	<0.001
Age squared	0.06	0.04-0.08	<0.001
Hospital +	-2.1	-9.0,4.7	0.540
Calcar destruction ‡	-8.3	-13.9,-2.6	0.004
Age*calcar destruction ‡	-0.8	-1.2,-0.4	<0.001
>25 % of femoral area involved ‡	-13.2	-0.2,-26.2	0.046
Age*>25% of femoral area involved ‡	-1.2	-1.8,-0.5	0.001
Radiolucency ‡	-3.9	-6.9,-1.0	0.009
Bilateral involvement ‡	-9.8	-17.2,-2.4	0.010
Increased bone turnover ‡	1.1	-5.5,7.9	0.735
Growth hormone excess ‡	-7.2	-12.8,-1.5	0.013
Hyperthyroidism ‡	-11.3	-16.9,-5.6	<0.001
FGF23-mediated hypophosphatemia ‡	-3.8	- 9.9,2.4	0.230

+ Reference category is NIH , ‡ Reference category is absence of risk factor

effects of phosphate or vitamin D supplementation/bisphosphonates/denosumab could not be assessed. Lastly, the study may be subjected to selection bias, as asymptomatic patients may not have been referred to the two tertiary referral centers. Yet, since many patients with limited disease (monostotic disease, lesions extending to <25% of the femoral area) and without deformity were included in our cohort, it presumably did not affect the risk factor analyses. In our opinion the inclusion of patients with low disease burden enabled an adequate execution of the nested case-control analyses and the linear mixed effects model. Nevertheless, the prevalence of coxa vara may not be generalizable to the total population of patients with FD/MAS. Similarly, we were merely able to calculate the proportion of patients with the subtypes of FD/MAS within the group with deformity, but not the absolute risk of deformity for every subtype of FD/MAS, due to the possible selection bias. Future studies should evaluate effects of treatment of the identified risk factors according to our proposed guideline on deformity progression and clinical outcomes, or investigate new therapeutic strategies targeting disease progression at early age.

In conclusion, in tertiary care centers one-third of femurs affected with FD in the proximal part develop coxa vara deformity. Risk factors include high percentage of the femur affected, calcar destruction, radiolucency and a diagnosis of McCune-Albright syndrome, specifically hyperthyroidism and growth hormone excess and possibly FGF23-mediated hypophosphatemia. Patients at risk

should be monitored yearly with radiographic assessment of the NSA and risk factors, and should receive medical treatment for endocrinopathies and to optimize bone quality. Deterioration accelerates if the NSA declines <120° before age 15, in which case surgical intervention must be considered.

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Part 3

Treatment

Chapter 7

A multidisciplinary care pathway improves quality of life and reduces pain in patients with Fibrous dysplasia/McCune-Albright syndrome: a multicenter prospective observational study

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Abstract

Background: Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) may cause pain, impaired ambulation and decreased quality of life (QoL). International guidelines advocate management of FD/MAS in a tertiary multidisciplinary care pathway, but no longitudinal data are available to support this recommendation. This multicenter prospective observational study aimed to evaluate effects of 1 year of treatment in the FD/MAS care pathway in 2 tertiary clinics on QoL and pain, assessed by change in Short Form 36 and Brief Pain Inventory between baseline and follow-up. Patients completing baseline questionnaires <1 year after intake were classified as new referrals, others as under chronic care.

Results: 92 patients were included, 61 females (66%). 22 patients (24%) had monostotic disease, 16 (17%) isolated craniofacial FD, 27 (40%) polyostotic FD and 17 (19%) MAS. 26 were new referrals (28%) and 66 chronic patients (72%). Median age at baseline was 47 years (Q1-Q3 36-56). Skeletal burden correlated with baseline Physical Function (r_s=-0.281,p=0.007). QoL was in all domains lower compared to the general population. New referrals reported clinically important differences (CID) over time in domains Physical Function (mean 67±SD24 to 74±21, effect size (ES) 0.31, p=0.020), Role Physical (39±41 to 53±43, ES 0.35, p=0.066), Social Functioning (64±24 to 76±23, ES 0.49, p=0.054), and Health Change (39±19 to 53±24, ES 0.76, p=0.016), chronic patients in Physical Function (52±46 to 66±43, ES 0.31, p=0.023) and Emotional Wellbeing (54±27 to 70±15, ES 0.59, p<0.001). New referrals reported a CID of 1 point in maximum pain, average pain and pain interference, chronic patients reported stable scores. Change in pain interference and Role Physical were correlated (r_s=-0.472,p<0.001). Patients with limited disease extent improved more than patients with severe disease. Patients receiving FD-related therapy had lower baseline scores than patients not receiving therapy and reported improvements in QoL after 1 year. Yet also patients without FD-related therapy improved in Physical Function.

Conclusions: All FD-subtypes may induce pain and reduced QoL. A multidisciplinary care pathway for FD/MAS may improve pain and QoL, mainly in new referrals without MAS comorbidities with low baseline scores. Therefore we recommend referral of patients with all subtypes of FD/MAS to specialized academic centers.

Background

Fibrous dysplasia (FD), is a rare, congenital bone disease, where fibrous skeletal lesions develop due to postzygotic mutations in the GNAS gene and subsequent aberrant bone turnover[1, 2]. The genetic mosaicism leads to a variable range of skeletal involvement and to a heterogeneous presentation, ranging from a single affected bone (monostotic FD) to severe disease with multiple bones involved (polyostotic FD)[3]. Because of the asymptomatic subset of patients, the prevalence remains unknown. In symptomatic patients pain and impaired mobility are common, as well as susceptibility to fractures or deformity. Additional extraskeletal manifestations of the GNAS mutation may be present in the McCune-Albright Syndrome (MAS), including endocrinopathies and skin hyperpigmentation[3, 4]. Recently, management guidelines have been established by the FD/MAS international consortium to address the diagnostic and therapeutic challenges emerging from the rarity of the disease, complex multiorgan involvement and heterogeneous phenotype[5]. This guideline provides a care pathway for diagnosis, staging, monitoring and treatment to alleviate symptoms. The provided treatment protocols consist of general measures, pharmacological therapy and surgical interventions, and are based on expert opinion and published studies[5, 6]. Most of these studies address objective outcome measures such as bone turnover markers after medical therapy[7-10], or radiologic outcomes or revisions after surgery[11-13]. However, no studies have evaluated the effect of treatment on patient reported outcome measures (PROMs) or the longitudinal follow-up of PROMS in FD/MAS in general. Yet this topic cannot be neglected since patients with FD/MAS report impairments in several domains of quality of life (QoL) compared to the general population[14] and more negative illness perceptions[15, 16]. These psychosocial consequences correlate with disease severity[14-16], but may also be influenced by inadequate information, misdiagnosis, diagnostic delay or ineffective treatment, problems which arise frequently due to the rarity and heterogeneity of FD/MAS[5]. In addition, the follow-up of PROMs is important to support the recommendation to manage FD/MAS in a specialized center. The Leiden University Medical Center (LUMC) and Radboud University Medical Center (RUMC) are both tertiary referral centers for FD/MAS and have implemented the care pathway as proposed in the guidelines, to tackle all physical and psychosocial aspects of FD/MAS, to minimize diagnostic and therapeutic delay, and to improve (patient reported) outcomes. Patients are provided with information and counselling and the diagnostic and therapeutic strategy advocated in the guidelines are coordinated in a multidisciplinary setting. Care pathways have been successful for other diseases and have shown to provide enhanced adherence to guidelines and documentation of care[17-19], reduced variability in care[20, 21], improved clinical outcomes[18, 20-23] and better teamwork[24].

For FD/MAS the value of a care pathway has not yet been established. For this reason we aimed to evaluate the effect of the LUMC/RUMC multidisciplinary pathway on QoL and pain in patients with FD/MAS, as we expected improvements compared to usual care. Secondary aims were to compare QoL with the general population, to assess differences between FD subtypes, to compare patients with and without FD-related therapy during follow-up and evaluate illness perceptions.

Methods

Population

This study was conducted between January 2018 and August 2021 and was performed within the PROFID-study, an ongoing multicenter prospective observational cohort study into patients with FD/MAS. The PROFID started in 2017 and is conducted on the outpatient clinics of Endocrinology and Orthopaedic Surgery of the LUMC and RUMC. Inclusion criteria are certain diagnosis FD/MAS (monostotic FD, polyostotic FD or MAS) and at least 1 visit at one of the outpatients clinics. In the current cohort we only presented result of the adult population. Written informed consent is required for inclusion. The study was approved by the Medical Ethics Committee of both centers (protocol number P17.136). Several patients have been included in previous studies[14-16, 25, 26], but data were not reused and generated originally for this study.

Research materials

Patients were asked to complete questionnaires upon inclusion in the study and again after 1 year of care in the FD/MAS care pathway. If follow-up visits were not deemed necessary or were planned further ahead than 1 year, questionnaires were send regardless of outpatient clinic visits, 1 year after completion of the baseline questionnaire. The questionnaire set included the Short Form 36 (SF-36)[27] and EuroQoL-5D-3L (EQ-5D)[28] for quality of life, the Brief Pain Inventory (BPI)[29] and the Illness Perceptions Questionnaire - Revised (IPQ-R)[30]. The SF-36 includes physical health domains (limitations in physical activities due to health problems ('Physical Function'), bodily pain and limitations in usual role activities due to physical health problems ('Role Physical')); mental health domains (vitality ('Energy/fatigue'), general mental health ('Emotional wellbeing'), limitations in usual role activities due to emotional problems ('Role Emotional'), limitations in social activities due to emotional or physical problems ('Social Functioning')); and general health domains (self-reported general health ('General health') and health change compared to 1 year ago). The range is 0-100 on each domain and higher scores reflect better QoL. This is similar for the EQ-5D

health state, where patients are asked to rate their health state at that moment on a visual analogue scale, 0 reflecting the worst possible health state and 100 the best state In addition, the EQ-5D comprises 5 subdomains on Mobility, Self-care, Usual Activities, Pain/Discomfort and Anxiety/Depression. Self-perceived problems can be scored on each domain on 3 levels: 1) no problems, 2) some problems, or 3) extreme problems. A summary score can be calculated with a publicly available formula that attaches weights to each level in each dimension. For this study the summary score and health state were used. The BPI is divided in a part on pain severity, where patients score their worst pain, least pain, pain on average and pain right now, and on interference of pain with functioning (e.g. general activity, mood, work). The range is 0-10, higher scores reflect more pain or more pain interference. We considered the items maximum pain, average pain and pain interference with general activity most relevant for our research and excluded other domains. For the IPQ-R the range is 6-30 for Timeline Acute/Chronic, Consequences, Personal Control and Emotional Representation, 4-20 for timeline cyclical, 5-25 for treatment control and illness coherence. Higher scores for Personal/Treatment Control and Illness Coherence indicate positive beliefs about controllability and understanding of the disease, whereas higher scores on Timeline Acute/Chronic/Cyclical, on Consequences and on Emotional Response represent strong beliefs on the acute/chronic/cyclical nature and on negative consequences and emotions caused by the disease. Baseline characteristics including FD subtype, FD-related comorbidity, skeletal burden score (SBS)[31] and treatment history were retrieved from electronic health records.

Research methods

For this study patients were selected who completed at least one questionnaire at both time points. The primary endpoint was the change in SF-36 and BPI scores during the 1 year of follow-up. This endpoint is assessed in 2 groups: patients were regarded as new referrals if the baseline questionnaire was completed within 1 year after intake in the hospital and all other patients were classified as under chronic care. This distinction was made because in the newly referred group, the baseline scores for QoL and pain can be used as proxy for the level of QoL and pain at the end of the care trajectory elsewhere. In this way the temporal change during follow-up was regarded to reflect the added value of the FD/MAS care pathway in comparison to usual care. Change in QoL and pain in chronic patients was assessed to observe baseline differences between groups and longitudinal change in this subgroup. For the SF-36 the clinically important difference (CID) was expressed as effect size (ES) and a threshold of 0.20 was used[32], for the BPI the CID was considered 1 point reduction[33]. Secondary endpoints were differences in SF-36 scores between the FD/MAS cohort and the general population, temporal change in EQ-5D scores, baseline and

temporal differences in SF-36 and pain scores between FD subtypes (monostotic non-craniofacial FD (MFD), isolated craniofacial FD (CFD), PFD and MAS) and between treatment groups during follow-up (FD-related therapy during follow-up or no therapy), and lastly differences in IPQ-R scores between referral groups and over time.

Diagnostic and therapeutic protocol of the FD/MAS care pathway

Patients are referred to the LUMC or RUMC by general practitioners, non-academic hospitals or academic hospitals. The diagnosis, often established in the referring center, is made on clinical, biochemical, radiographical or pathological parameters. During the 1 year of follow-up in this study patients were staged and treated in the FD/MAS care pathway according to the guidelines[5]. The FD/MAS team consists of endocrinologists and orthopaedic surgeons who are experienced and specialized in FD/MAS. Close multidisciplinary collaboration allows for comprehensive screening of the patient; for a rapid management plan; and for thorough provision of background information with counselling and reassurance. Patients are screened for complaints, skeletal complications or additional endocrinopathies. Bone-related laboratory testing is performed upon intake, during active surveillance, and before and after treatment. Imaging of the lesions is conducted and skeletal scintigraphy or ^{18}F-NaF PET/CT[34] to determine disease extent. If indicated, a patient-tailored assessment of vision, hearing, endocrine function or other extraskeletal morbidity is conducted with consultation of relevant specialists including ophthalmologists, oral- and maxillofacial surgeons, neurosurgeons and rehabilitation physicians. Asymptomatic patients remain under surveillance unless wishing to return to the referring center. Symptomatic patients may be treated with physical therapy or rehabilitation; with medical therapy such as analgesics, supplements of phosphate/vitamin D/calcium, bisphosphates or as a last resort denosumab; or with surgery for mechanical pain, (impending) fracture, deformity or nerve compression. A detailed description is provided in the guidelines[5].

Statistical analysis

Categorical data are presented as number (percentage), numerical data as mean (±SD) or median (quartile 1-quartile 3). SF-36 scores were compared with the general Dutch population by one-sample t-tests. Numerical data were in case of normality compared between time points with paired t-tests, between 2 groups with independent t-tests, and between the 4 FD subtypes by one-way ANOVA, whereas in absence of normality Wilcoxon Signed rank, Mann-Whitney U and Kruskal Wallis tests were used respectively. Categorical data were compared with Chi Square tests. Analyses concerning pain were conducted in

patients with pain (baseline maximum or average pain score ≥0) and with moderate to severe pain (score ≥4/10), because the pain severity in the latter group is an indication for analgesic therapy. Subgroups for FD-related treatment during follow-up were compared on the presence or absence of antiresorptive or surgical treatment during the 1 year in the care pathway. The correlation between SBS and SF-36 domain Physical Function at baseline was assessed by Spearman's rank-order correlation, as well as the correlation between temporal change in pain interference and in Role Physical. Correction for multiple testing was not performed because of the exploratory nature of the study, because the CID was deemed more important, and to omit the misuse of the p-value as dichotomizing instrument[35, 36]. Missing data were excluded and analysed by comparing characteristics of complete cases to patients with no questionnaires or questionnaires completed a merely 1 time point.

Results

Patient characteristics

92 consecutive patients were included in this study, 26 new referrals (28.3%) and 66 patients under chronic care (72.7%) (table 1). 85 patients (92%) completed all questionnaires and 7 patients (8%) filled in questionnaires at both time points but incompletely. Median age at baseline was 46.5 (Q1- Q3 36-56) and comparable between referral groups. The total cohort consisted of 61 females (66.3%). 22 patients (24%) were diagnosed with MFD, 16 (17%) with CFD), 27 (40%) with PFD and 17 (19%) with MAS. Patients in the chronic group were more frequently affected with PFD or MAS.

Median SBS was 3.2 (1.2-16.9) and comparable between groups. SBS correlated with SF-36 domain Physical Function at baseline (Spearman's rho = - 0.281, p=0.007). Most patients were included in the LUMC (n=77, 83.7%) and were referred from a non-academic hospital (n=42, 46%) or from another academic hospital (n=23, 25%). Mean follow-up (time between survey completion) was 1.2 (±SD 0.3) years. The majority of patients had received FD-related treatment prior to inclusion in this study, specifically bisphosphonates (n=68, 74%), denosumab (n=20, 22%) or surgery (n=40, 44%). Half of the patients had received FD-related treatment during follow-up: 35 patients (38%) received bisphosphonates, 16 (17%) denosumab and 5 (5%) surgery, whereas 42 patients (46%) did not receive FD-related therapy.

Table 1. Baseline characteristics

	New (n=26)	Chronic (n=66)	Total (n=92)
Age at baseline years, median (Q1-Q3)	47.5 (28-59)	46 (37-55)	46.5 (36-56)
Sex, female n (%)	11 (42%)	50 (76%)	61 (66.3%)
Type of FD [a] n (%)			
MFD (non-CFD)	9 (34.6%)	13 (19.7%)	22 (23.9%)
Isolated CFD	7 (26.9%)	9 (13.6%)	16 (17.4%)
PFD	8 (30.8%)	29 (43.9%)	37 (40.2%)
MAS	2 (7.7%)	15 (22.7%)	17 (18.5%)
Skeletal Burden Score [b] median (Q1-Q3)	3.2 (0.5-15.8)	4.8 (1.2-17.1)	3.2 (1.2-16.9)
Hospital n (%)			
LUMC [c]	25 (96.2%)	52 (78.8%)	77 (83.7%)
RUMC [d]	1 (3.8%)	14 (21.2%)	15 (16.3%)
Referring physician n (%)			
General practitioner	4 (15.4%)	10 (15.2%)	14 (15.2%)
Non-academic hospital	14 (53.8%)	28 (42.4%)	42 (45.7%)
Academic hospital	7 (26.9%)	16 (24.2%)	23 (25%)
Unknown	1 (3.8%)	12 (18.2%)	13 (14.1%)
Time between surveys years, mean (±SD)	1.3 (0.4)	1.2 (0.3)	1.2 (0.3)
Treatment history prior to inclusion n (%)			
Bisphosphonates	16 (61.5%)	52 (78.8%)	68 (73.9%)
Denosumab	1 (3.8%)	19 (28.8%)	20 (21.7%)
Surgery	7 (26.9%)	33 (50%)	40 (43.5%)
None of the above	9 (34.6%)	8 (12.1%)	17 (18.5%)
Treatment during follow-up n (%)			
Bisphosphonates	15 (57.7%)	20 (30.3%)	35 (38%)
Denosumab	1 (3.8%)	15 (22.7%)	16 (17.4%)
Surgery	2 (7.7%)	3 (4.5%)	5 (5.4%)
None of the above	10 (38.5%)	a32 (48.5%)	42 (45.7)

a: MFD: monostotic fibrous dysplasia, CFD: craniofacial FD, PFD: polyostotic FD, MAS: McCune-Albright syndrome. b: range: 0-100. c: LUMC: Leiden University Medical Center. d: RUMC: Radboud University Medical Center

Primary outcome – temporal change in SF-36 scores in new referrals and chronic patients

New referrals reported clinically important improvements in Physical Function after 1 year of follow-up, from mean 67 (±SD 24) to 74 (±21) (p=0.020) (table 2, fig. 1). Chronic patients reported slightly higher Physical Function at baseline compared to new referrals, but no improvements during follow-up, resulting in similar scores after 1 year (table 2, figure 1). Role Physical was at baseline higher in chronic patients and in both groups a clinically relevant improvement of 14 points was observed. Domains Pain and Energy/Fatigue were in both groups similar at baseline and stable over time. Emotional Wellbeing was comparable between groups at baseline and improved more in chronic patients, from 54 (±27) to 70 (±15) (p<0.001), compared to new referrals, 56 (±21) to 60 (±17)

(p=0.275). Social Functioning improved in new referrals from 64 (±24) to 76 (±23) (p=0.054), was in chronic patients higher at baseline but stable over time, and was therefore comparable between groups after 1 year. Role Emotional and General Health were at baseline higher in chronic patients and in both groups stable over time. In contrast Health Change improved in new referrals from 39 (±19) to 53 (±24) (p=0.016), whereas chronic patients reported no change.

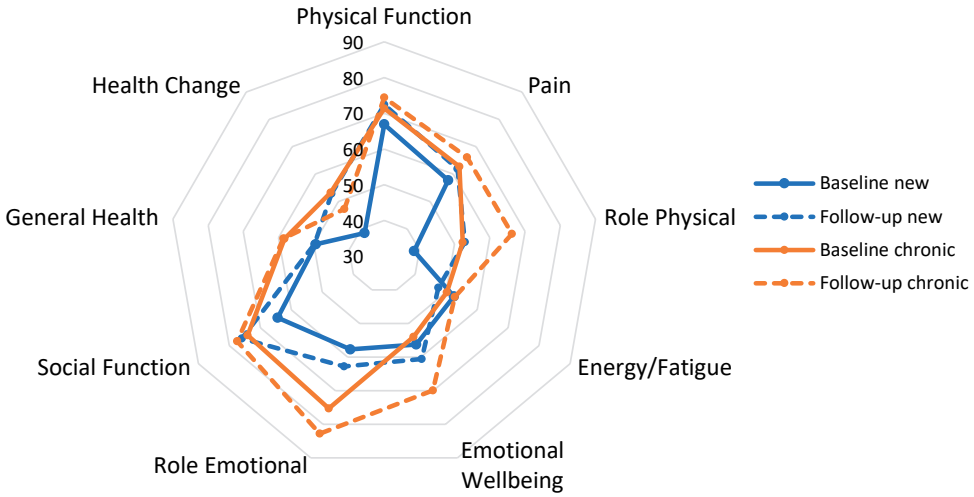

Figure 1. SF-36 domains at baseline and follow-up in new and chronic patients

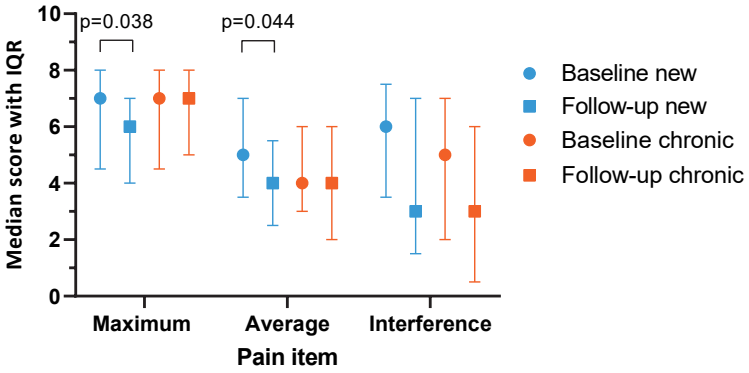

Figure 2. Pain scores at baseline and follow-up in new and chronic patients with moderate to severe pain

Table 2. Quality of life scores of Short Form 36 *

	Baseline mean (±SD)	**Follow up mean (±SD)**	**Difference mean (95% CI)**	**Effect Size**	**p-value [a]**
		Physical function			
New (n=26)	66.9 (23.6)	74.2 (20.9)	+ 7.3 (1.2-13.4)	0.31 [+]	p=0.020
Chronic (n=66)	71.4 (24.6)	74.4 (23.3)	+ 3.0 (- 0.61-6.5)	0.12	p=0.102
Difference mean (95% CI)	4.5 (- 6.7-15.7)	0.2 (-10.3-10.6)			
p-value [b]	p=0.425	p=0.975			
		Pain			
New (n=26)	57.8 (25.9)	61.9 (23.7)	+ 4.1 (- 4.9-13.1)	0.16	p=0.360
Chronic (n=66)	62.7 (26.3)	66.1 (25.2)	+ 3.3 (- 2.4-9.1)	0.13	p=0.251
Difference mean (95% CI)	5.0 (- 7.1-17.0)	4.2 (- 7.2-15.6)			
p-value [b]	p=0.415	p=0.463			
		Role limitations due to physical problems			
New (n=26)	38.5 (40.8)	52.9 (43.2)	+ 14.4 (- 1-29.8)	0.35 [+]	p=0.066
Chronic (n=66)	52.3 (45.5)	66.3 (43.1)	+ 14.0 (2.0-26.1)	0.31 [+]	p=0.023
Difference mean (95% CI)	13.8 (- 5.8-33.4)	13.4 (- 6.4-33.2)			
p-value [b]	p=0.163	p=0.183			
		Energy/fatigue			
New (n=26)	52.5 (20.6)	47.5 (15.9)	- 5.0 (- 12.8-2.8)	0.24 [+]	p=0.199
Chronic (n=66)	50.2 (20)	52.8 (17.8)	+ 2.6 (- 3.0-8.1)	0.13	p=0.358
Difference mean (95% CI)	2.3 (- 11.5-7.0)	5.3 (- 2.7-13.3)			
p-value [b]	p=0.627	p=0.189			
		Emotional wellbeing			
New (n=26)	56.3 (20.7)	60.6 (16.6)	+ 4.3 (- 3.7-12.3)	0.21 [+]	p=0.275
Chronic (n=66)	54.0 (27.1)	69.9 (15.1)	+ 15.9 (9.2-22.7)	0.59 [+]	p<0.001
Difference mean (95% CI)	- 2.3 (- 12.8-8.2)	9.3 (2.2-16.5)			
p-value [b]	p=0.662	p=0.011			
		Role limitations due to emotional problems			
New (n=26)	57.7 (42.7)	62.8 (44.5)	+ 5.1 (- 14.2-24.4)	0.12	p=0.589
Chronic (n=66)	75.3 (36.2)	82.8 (32.7)	+ 7.6 (- 2.0-17.2)	0.21 [+]	p=0.121
Difference mean (95% CI)	17.6 (0.2-35.1)	20.0 (0.5-39.5)			
p-value [b]	p=0.050	p=0.045			
		Social Functioning			
New (n=26)	64.4 (23.6)	76.0 (22.6)	+ 11.5 (- 0.3-23.3)	0.49 [+]	p=0.054
Chronic (n=66)	74.1 (24.2)	77.5 (21.4)	+ 3.4 (- 1.2-8.0)	0.14	p=0.146
Difference mean (95% CI)	9.6 (- 1.5-20.7)	1.5 (- 8.5-11.5)			
p-value [b]	p=0.088	p=0.766			
		General Health			
New (n=26)	49.4 (20.9)	49.8 (17.4)	+ 0.4 (- 6.1-6.9)	0.02	p=0.903
Chronic (n=66)	58.3 (20.6)	58.6 (19.9)	+ 0.2 (- 3.0-3.5)	0.01	p=0.889

Table 2. Quality of life scores of Short Form 36 * (*continued*)

	Baseline mean (±SD)	**Follow up mean (±SD)**	**Difference mean (95% CI)**	**Effect Size**	**p-value [a]**
Difference mean (95% CI)	8.9 (- 0.6-18.4)	8.8 (- 0.1-17.6)			
p-value [b]	p=0.066	p=0.053			
Health change compared to 1 year ago					
New (n=26)	38.5 (19.0)	52.9 (23.8)	+ 14.4 (2.9-25.9)	0.76 [+]	p=0.016
Chronic (n=66)	48.1 (21.6)	47.3 (19.2)	- 0.76 (- 7.9-6.4)	0.03	p=0.833
Difference mean (95% CI)	9.6 (0.0-19.3)	-5.5 (- 15-3.9)			
p-value [b]	p=0.050	p=0.248			

*: Higher scores reflect better QoL. Range: 0-100. a: Paired samples t-test, b: independent samples t-test. +: Clinically important difference, effect size above threshold of 0.20

Primary outcome – temporal change in BPI scores in new referrals and chronic patients

In the combined cohort 62 patients (67%) reported moderate to severe pain at baseline. New referrals in this subgroup reported a decreased of 1 point (Q1-Q3 -0.5-3) in maximum pain (p=0.038), 1 point (0-2.5) in average pain (p=0.044) and 1 point (- 0.5-3) in interference of pain with general activity, which decreased from median 6 (3.5-7.5) to 3 (1.5-7) (p=0.090) (table 3, fig. 2). In chronic patients with moderate to severe pain, scores did not change for maximum and average pain, but pain interference improved from 5 (2-7) to 3 (0.5-6) (p=0.083) although median change was 0 (- 2.5-1) (table 3, figure 2). The improvements in new referrals resulted in comparable scores in both groups after 1 year for average pain and pain interference. In new referrals reporting pain (score >0), a decrease of 1 point was also observed in all 3 pain items (table 4, fig 3). The largest improvement was observed in pain interference from median 5 (2-7) to 2 (1-7) (p=0.099). Chronic patients had better scores at baseline and no temporal change, hence scores were similar in both groups after 1 year. In the total cohort a moderate correlation was observed between temporal change in pain interference and in SF-36 domain Role Physical (Spearman's rho = - 0.472, p<0.001).

Secondary outcome - comparison of SF-36 with general population

In the total cohort significant and clinically relevant impairments in all domains of QoL were observed compared to the general Dutch population[37] (Additional file 1, suppl. table A and figure A). Differences were most outspoken in the domains Emotional Wellbeing (FD/MAS 55 ±25, population 77 ±17, p<0.001), Energy/Fatigue (FD/MAS 51 ±20, population 69 ±19, p<0.001), Role Physical (FD/MAS 49 ±44, population 76 ±36, p<0.001) and General Health (FD/MAS 56 ±21,

population 71 ±21, p<0.001). After 1 year scores improved in several domains but did not reach the level of the general population.

Table 3. Pain scores of Brief Pain Inventory in new and chronic patients with moderate to severe pain *

	Baseline median (Q1-Q3)	**Follow up median (Q1-Q3)**	**Difference median (Q1-Q3)**	**p-value [a]**
		Maximum pain		
Total (n=62)	7 (4.75-8)	6 (4-8)	0 (- 2-1)	p=0.077
New (n=21)	7 (4.5-8)	6 (4-7)	- 1 (- 3-0.5)	p=0.038
Chronic (n=41)	7 (4.5-8)	7 (5-8)	0 (- 2-1)	p=0.512
p-value [b]	p=0.976	p=0.132		
		Average pain		
Total (n=62)	4.5 (3-6)	4 (2-6)	0 (- 2-1)	p=0.015
New (n=21)	5 (3.5-7)	4 (2.5-5.5)	- 1 (- 2.5-0)	p=0.044
Chronic (n=41)	4 (3-6)	4 (2-6)	0 (- 1.5-1)	p=0.140
p-value [b]	p=0.178	p=0.976		
		Interference of pain with general activity		
Total (n=62)	5 (2.75-7)	3 (1-6)	- 1 (- 2.25-1)	p=0.016
New (n=21)	6 (3.5-7.5)	3 (1.5-7)	- 1 (- 3-0.5)	p=0.090
Chronic (n=41)	5 (2-7)	3 (0.5-6)	0 (- 2.5-1)	p=0.083
p-value [b]	p=0.284	p=0.674		

*: in patients with moderate to severe pain at baseline (maximum or average pain >4). Higher scores = more pain and more interference. Range: 0-10. a: Wilcoxon signed rank test, b: Mann Whitney U test

Secondary outcome – temporal change in EQ-5D

In new referrals the EQ-5D health state increased from median 68 (Q1-Q3 44-90) to 80 (59-91), p=0.088. The summary score and all scores for chronic patients remained constant (data not shown).

Secondary outcome – QoL across FD subtypes

Patients with isolated CFD reported better Physical Function (84 ±SD23) compared to patients with MFD (63 ±31), PFD (70 ±19) or MAS (67 ±24) (p=0.050) (Add. file 1, suppl. table B & fig. B). Physical Function improved during follow-up in all subtypes except for MAS. Scores for the Pain subscale were comparable across subtypes and also improved least in MAS. In patients with MAS Role Physical was least experienced at baseline and stable over time. On the contrary all other subtypes reported improvements in Role Physical by 17-21 points during follow-up. In domain Energy/Fatigue patients with PFD scored worst at baseline (47 ±20) but experienced most improvement at follow-up (54 ±26) (p=0.013).

Table 4. Pain measured with Brief Pain Inventory in patients with pain score >0 *

	Baseline median (Q1-Q3)	Follow up median (Q1-Q3)	Difference median (Q1-Q3)	p-value [a]
		Maximum pain		
Total (n=84)	6 (3-7.75)	5 (3-7)	0 (- 2-1)	p=0.656
New (n=23)	7 (4-8)	5 (4-7)	- 1 (- 3-1)	p=0.074
Chronic (n=61)	5 (2-7)	5 (3-7)	0 (- 1-1.5)	p=0.519
p-value [b]	p=0.078	p=0.984		
		Average pain		
Total (n=84)	3.5 (2-5)	3 (2-5)	0 (- 1-1)	p=0.174
New (n=23)	5 (3-7)	4 (2-5)	- 1 (- 2-0)	p=0.044
Chronic (n=61)	3 (2-5)	3 (2-5)	0 (- 1-1)	p=0.816
p-value [b]	p=0.013	p=0.514		
		Interference of pain with general activity		
Total (n=84)	4 (1-7)	2 (0.25-6)	0 (- 2-1)	p=0.080
New (n=23)	5 (2-7)	2 (1-7)	- 1 (- 2-1)	p=0.099
Chronic (n=61)	3 (0-6)	2 (0-5)	0 (- 2-1)	p=0.326
p-value [b]	p=0.072	p=0.363		

*: in patients with pain at baseline (maximum or average pain >0). Higher scores = more pain and more interference. Range 0-10 a: Wilcoxon signed rank test, b: Mann Whitney U test

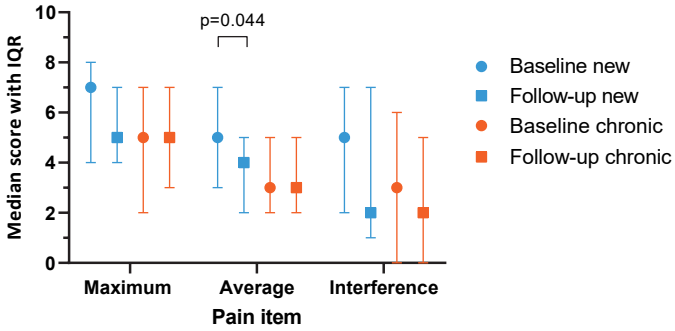

Figure 3. Pain scores at baseline and follow-up in new and chronic patients with pain score >0

In other FD subtypes scores remained stable. Emotional Wellbeing was most impaired in patients with MFD (49 ±29) and MAS (50 ±29) and improved in both groups to 63 (±19) (p=0.05) and to 71 (±11) (p=0.017) respectively. Also patients with PFD reported higher scores for Emotional Wellbeing at follow-up. Role Emotional was similar between subtypes and time points. Social Functioning was most impaired in MFD and CFD and improved in all subtypes except MAS.

General Health did not differ between FD subtypes and no improvements were observed at follow-up. All patients except for those with MAS reported slightly better health than 1 year ago at follow-up than at baseline.

Secondary outcome - pain across FD subtypes

In patients with moderate to severe pain, for whom analgesic therapy is indicated, scores for maximum pain were comparable across FD subtypes (Add. file 1, suppl. table C, fig C). Average pain was least severe in patients with CFD (median 3, Q1-Q3 3-4) and most in MFD (5, 3-7) and MAS (5, 3-6), p=0.165. Similarly pain interference was least experienced in CFD (4, 2-7) and most in MFD (7, 4-8). In patients reporting pain with score >0, patients with PFD reported the highest score for maximum pain of 7 (3-7.5), versus 5 (2.5-8) in patients with MFD, 5.5 (2-7) for CFD and 5 (1-8) for MAS (Add. file 1, suppl. table D & fig. D). Average pain scores were comparable across diagnoses, and pain interference was least experienced by patients with CFD and MAS (both median 2.5) compared to patients with MFD and PFD (median 4).

Secondary outcome – QoL for treatment received during follow-up

In the entire cohort 50 patients (54.3%) received FD-related therapy during the 1 year of follow-up (antiresorptive therapy: n=45, 48%, surgery: n=5, 5%) (Add. file 1, suppl. table E & fig. E). In patients receiving therapy Physical Function was lower at baseline (65 ±24) compared to patients receiving no therapy (77 ±23, p=0.041), but improved during follow-up to 71 (±24) (p=0.007). Similarly scores for Role Physical were lower in patients receiving treatment, although in this domain remarkably the greatest improvement was observed in patients not receiving therapy (55 ±44 to 74 ±38, p=0.004, ES 0.47). Emotional Wellbeing and Social Functioning were both lower at baseline but improving during follow-up in patients with FD-related therapy.

Secondary outcome – pain for treatment received during follow-up

Patients receiving FD-related therapy during follow-up had worse scores for maximum pain, average pain and pain interference at baseline compared to patients without therapy (Add. file 1, suppl. table F & fig F), as expected since it was part of the treatment indication. In the therapy group the median of all scores decreased slightly during follow-up, mainly pain interference from 5 (2-7) to 3 (1-6) (=p=0.061), but with median difference of 0. No change was observed in patients without FD-related therapy.

Secondary outcome – temporal change in illness perceptions

In general scores for illness perceptions were comparable over time for both new referrals and chronic patients (Add. file 1, suppl. table G). No differences were observed between FD subtypes.

Missing data analysis

The 92 patients with questionnaires at both time points were retrieved from a cohort of 124 patients who were included in the PROFID study before August 1st 2020 and received both questionnaires, providing a response rate of 74%. 26 of the excluded patients completed only the first questionnaire (21% of total) and 6 patients (5%) completed none after 2 reminders. Complete cases were slightly older than excluded patients, but the sex ratio and FD subtype were comparable between groups (appendix table G). Completers did not differ from dropouts in baseline QoL or pain (suppl. table H).

Discussion

This study aimed to examine the effect of a coordinated care pathway, with information provision, counseling, and a multidisciplinary care as described in the guidelines, on QoL and pain in patients with FD/MAS. At baseline patients with FD/MAS reported significant and clinically relevant impairments in all domains of QoL and the majority reported moderate to severe pain. After referral to our tertiary FD clinics, patients reported a clinically important difference in SF-36 domains Physical Function, Role Physical, Social Functioning and Health Change as well as a clinically relevant decrease of 1 point in maximum pain, average pain and pain interference. The baseline scores in our study reflect the level of QoL and pain accomplished during usual care in the referring secondary care facility, as the questionnaires were completed shortly after referral to our hospital prior to treatment, and therefore these data suggest that treatment in our tertiary FD care pathway for FD/MAS may benefit QoL and pain compared to usual care in other hospitals. Remarkably the SF-36 domain Pain did not reflect the improvements observed in the BPI. This SF-36 domain consists of 2 questions on pain intensity in 6 response categories and pain interference in 5. This scale may not have been sufficiently responsive to change to capture the 1-point change demonstrated by the BPI. Yet the improvements in the domains interference of pain with general activity and in Role Physical appeared to be larger than in pain and Physical Function, which implies that the FD/MAS care pathway mainly improves negative consequences of pain and impaired mobility rather than the item itself. The moderate correlation between change in pain inference and in Role Physical domain underlines this observation. Another

striking finding is the discrepancy between stable scores for General Health and clinically relevant increases in Health Change. The General Health domain consists of items on self-perceived health status, tendency to become ill compared to others and expectations on future health whereas the Health Change domain consists of 1 item on health status compared to 1 year ago. Possibly health state is perceived as better than 1 year ago, but still considerably worse compared to others. Nevertheless this rise in Health Change is in line with the reported improvements in other domains and these data highlight that symptomatic patients with FD/MAS may benefit from referral to a specialized tertiary center with multidisciplinary care.

New referrals generally reported worse scores for QoL and pain at baseline compared to chronic patients. Yet also in chronic patients clinically relevant improvements were observed, specifically in Role Physical and Emotional Well-being. Similarly in several other SF-36 domains and in pain interference smaller improvements were observed, which were neither significant nor clinically relevant, but we cannot rule out that a larger difference may be accomplished during a longer trajectory in the FD/MAS care pathway. The amelioration in chronic patients, although to a lesser extent, suggests that the improvements in new referrals could not merely be attributed to low baseline performance with large room for improvement or to regression to the mean, but confirms that the care pathway benefits QoL and pain, even in long-term care.

The COVID-19 pandemic might have influenced our results, as half of the patients (n=49, 53%) completed the first questionnaire before and the second after the national social distancing measures in March 2020, whereas 22 patients (24%) completed both questionnaires before and 21 (23%) both during the pandemic. The major increase in Social Functioning, which we had not expected, may be explained by the fact that patients with low baseline scores for Social Functioning, mainly new referrals, experienced less difficulties in social life during the pandemic because of the absence of social gatherings.

A previous cross-sectional study on QoL and pain in FD/MAS has been conducted in our center by Majoor et al[14] and showed better scores for QoL and pain compared to the present study. Accordingly our cohort demonstrated impairments in all domains of QoL in comparison to the general Dutch population, whereas Majoor et al. did not detect this for domains Role Emotional and Mental Health. Since QoL and pain are affected by FD subtype, skeletal burden, lesion location and age[14, 25, 38], variable study populations will result in a diversity of QoL and pain scores. Our study consisted of more patients with PFD and MAS, less with MFD and included an extra subgroup of patients with CFD. Secondly our

study included new referrals, who may have a less extensive treatment history compared to patients under chronic care. Lastly mainly patients with MFD reported impairments in our study compared to the previous study, which may all account for the difference between both studies.

Indeed of all FD subtypes in our cohort, patients with MFD demonstrated most impairments in domains Physical Function, Pain and Role Physical and most interference of pain with general activities. Both QoL and pain responded well to academic care and improved during follow-up. The largest reduction in pain interference with general activity was observed in patients with isolated craniofacial FD. On the contrary patients with MAS experienced least improvement over time in both SF-36 and BPI scores. A hypothesis for the least severe FD subtypes benefiting most from therapy is that selection bias occurred when MFD or CFD patients with minor complaints were treated in non-academic centers and those with severe complaints were referred and treated in academic centers, selecting the patients who are more likely to benefit from treatment. Nevertheless this highlights that even patients with low skeletal burden may experience substantial negative FD-related consequences and may benefit from referral to a specialized center.

A secondary aim was to assess illness perceptions. The observed increase in QoL in our cohort could not be supported by a change in Illness Perceptions. The counselling, providing information and treatment in the LUMC care pathway surprisingly did not effectuate better perceived coherence of the disease or a higher perceived control over disease related complaints. The correlation between temporal change in SF-36 domain Role Physical and in pain interference is in line with the finding that QoL in our cohort was not influenced by altered illness perceptions but rather by improved pain management.

However QoL may even improve without change in pain scores. This was demonstrated by patients who had not received FD-related therapy during follow-up but still experienced a major improvement in Role Physical with an ES of 0.47 after 1 year, despite constant pain severity or interference. We hypothesize that the rise in QoL in our cohort may not only be explained by the effect of antiresorptive therapy or pain management but also by other factors, possibly multidisciplinary care, recognition of FD-related complaints, lifestyle advice or disease acceptance.

The COVID-19 pandemic could comprise a limitation of our study, as several negative consequences of the pandemic have been established in the general population including stress or worry[39], less physical activity[40], depressive symp-

toms, listlessness and decreased quality of life[41]. We were not able to assess the influence of several lockdowns on our study, but it might have exerted negative effects on Role Physical, Emotional Wellbeing, Energy/Fatigue, pain and pain interference. If so, this would result in an underestimation of the effect of the care pathway. Contrarily less physical activity could also benefit pain severity and interference. Another limitation is the discrepancy in sample sizes between referral groups, as the smaller sample of new referrals delivers results with more variance and more vulnerable to outliers. In addition the baseline scores of new referrals were used as proxy for the level of QoL and pain accomplished during secondary care before referral to our facility, but the true scores remain unknown. Finally our study comprised a heterogenous population of patients with FD/MAS with several subtypes, variable treatment history and various treatments started during follow-up. Ideally to assess the added value of the care pathway a randomized controlled trial should be conducted, where patients are randomized into treatment in the care pathway or in usual care, but limited resources hindered this design. Alternatively the approach in this study could be regarded as a strength, as it reflects the clinical heterogeneity present in every tertiary referral center and maintains external validity. This validity is further supported by the limited non-response bias, demonstrated by comparable characteristics of complete cases and non-responders. For these reasons results of this study may be generalized to patients with FD/MAS in other academic hospitals. Another strength of this study is the insight into questionnaires valuable for patients with FD/MAS, to screen for impairments not routinely addressed during standard medical consultations. Results were discussed with the Dutch Patient Association Fibrous Dysplasia to allow for improved and simplified questionnaire logistics in our FD/MAS care pathway. Lastly our study is the first to generate a longitudinal follow-up of PROMs in academic care and the first to combine outcomes such as QoL, mobility, pain and illness perceptions in patients with FD/MAS.

Conclusion

We have established that patients with all subtypes of FD/MAS may suffer from negative consequences of their disease, and that the multidisciplinary coordinated care pathway for FD/MAS may improve QoL, pain and in particular pain interference, even though illness perceptions are unchanged. Patients who seem to benefit most from this care pathway are symptomatic new referrals without MAS comorbidities with low baseline performance. We recommend referral of patients with all subtypes of FD/MAS to tertiary academic centers and the implementation of the multidisciplinary care pathway for FD/MAS in those

centers. Future studies should aim to determine the effect of the FD/MAS care pathway on time to diagnosis or therapy initiation, adherence to guidelines and uniformity of care. Lastly attention is required for risk factors for an impaired QoL, for considerable pain interference, and for treatment-resistance in patients with FD/MAS.

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Supplementary files

Table A. Quality of life scores of Short Form 36 *

	Baseline mean (±SD)	Follow up mean (±SD)	Difference mean (95% CI)	p-value [a]
Physical function				
Total (n=92)	70.2 (24.3)	74.3 (22.5)	+ 4.2 (1.1-7.2)	p=0.008
General population	83.0 (22.8)			
Difference mean (95% CI)	12.8 (7.8-17.9)	8.7 (4.0-13.3)		
p-value [b] and effect size	p<0.001, ES 0.56	p<0.001, ES 0.38		
Pain				
Total (n=92)	61.3 (26.1)	64.9 (24.7)	+ 3.5 (1.2-8.3)	p=0.142
General population	74.9 (23.4)			
Difference mean (95% CI)	13.6 (8.1-19.0)	10.0 (4.9-15.1)		
p-value [b] and effect size	p<0.001, ES 0.58	p<0.001, ES 0.43		
Role limitations due to physical problems				
Total (n=92)	48.4 (44.4)	62.5 (43.3)	+ 14.1 (4.6-23.7)	p=0.004
General population	76.4 (36.3)			
Difference mean (95% CI)	28.0 (18.8-37.2)	13.9 (4.9-22.9)		
p-value [b] and effect size	p<0.001, ES 0.77	p=0.03, ES 0.38		
Energy/fatigue				
Total (n=92)	50.9 (20.1)	51.3 (17.4)	+ 0.4 (- 4.1-5.0)	p=0.849
General population	68.6 (19.3)			
Difference mean (95% CI)	17.7 (13.6-21.9)	17.3 (13.7-20.9)		
p-value [b] and effect size	p<0.001, ES 0.92	p<0.001, ES 0.90		
Emotional wellbeing				
Total (n=92)	54.7 (25.3)	67.3 (16.0)	+ 12.7 (7.3-18.0)	p<0.001
General population	76.8 (17.4)			
Difference mean (95% CI)	22.1 (16.9-27.4)	9.5 (6.1-12.8)		
p-value [b] and effect size	p<0.001, ES 1.27	p<0.001, ES 0.55		
Role limitations due to emotional problems				
Total (n=92)	70.3 (38.7)	77.2 (37.3)	+ 6.9 (- 1.7-15.5)	p=0.115
General population	82.3 (32.9)			
Difference mean (95% CI)	12.0 (4.0-20.0)	5.1 (- 2.6-12.8)		
p-value [b] and effect size	p=0.004, ES 0.36	p=0.191, ES 0.16		
Social Functioning				
Total (n=92)	71.3 (24.3)	77.0 (21.6)	+ 5.7 (1.1-10.3)	p=0.016
General population	84.0 (22.4)			
Difference mean (95% CI)	12.7 (7.6-17.7)	7.0 (2.5-11.4)		
p-value [b] and effect size	p<0.001, ES 0.57	p=0.03, ES 0.31		

Table A. Quality of life scores of Short Form 36 * (*continued*)

	Baseline mean (±SD)	Follow up mean (±SD)	Difference mean (95% CI)	p-value [a]
		General Health		
Total (n=92)	55.8 (21.0)	56.1 (19.6)	+ 0.3 (- 2.6-3.2)	p=0.852
General population	70.7 (20.7)			
Difference mean (95% CI)	14.9 (10.5-19.2)	14.6 (10.6-18.7)		
p-value [b] and effect size	p<0.001, ES 0.72	p<0.001, ES 0.71		

*: Higher scores reflect better QoL. Range: 0-100. a: Paired samples t-test, b: one-sample t-test

Table B. Quality of life scores of Short Form 36 across FD subtypes*

Subgroup	Baseline mean (±SD)	Follow up mean (±SD)	Difference (95% CI)	p-value [a]
		Physical function		
MFD (n=22)	63.2 (30.5)	70.5 (26.7)	+ 7.3 (- 1.1-15.6)	p=0.085
CFD (n=16)	84.4 (22.6)	89.1 (14.3)	+ 4.7 (- 1.2-10.6)	p=0.110
PFD (n=37)	69.9 (19.0)	74.9 (16.5)	+ 5.0 (0.7-9.2)	p=0.022
MAS (n=17)	66.5 (23.6)	64.4 (28.4)	- 2.1 (- 9.8-5.7)	p=0.582
p-value [b]	p=0.050	p=0.011		
		Pain		
MFD (n=22)	56.6 (30.7)	61.8 (27.5)	+ 5.2 (- 4.1-14.6)	p=0.262
CFD (n=16)	67.1 (24.2)	75.1 (23.3)	+ 8.0 (- 5.6-21.6)	p=0.227
PFD (n=37)	59.2 (26.2)	63.0 (24.0)	+ 3.8 (- 4.3-11.9)	p=0.345
MAS (n=17)	66.6 (21.2)	63.3 (23.5)	+ 3.4 (- 13.8-7.1)	p=0.505
p-value [b]	p=0.493	p=0.342		
		Role limitations due to physical problems		
MFD (n=22)	40.9 (45.3)	58.0 (42.5)	+ 17.0 (- 4.1-38.2)	p=0.109
CFD (n=16)	48.4 (45.2)	70.3 (40.0)	+ 21.9 (1.9-41.9)	p=0.034
PFD (n=37)	50.0 (44.5)	67.6 (43.2)	+ 17.6 (4.0-31.2)	p=0.013
MAS (n=17)	54.4 (45.3)	50.0 (47.6)	- 4.4 (- 35.0-26.2)	p=0.764
p-value [b]	p=0.810	p=0.448		
		Energy/fatigue		
MFD (n=22)	53.2 (20.2)	51.1 (19.8)	- 2.21 (- 12.7-8.6)	p=0.693
CFD (n=16)	55.6 (20.1)	49.1 (16.8)	- 6.6 (- 18.0-4.9)	p=0.241
PFD (n=37)	46.5 (19.7)	54.5 (16.4)	+ 8.0 (1.8-14.2)	p=0.013
MAS (n=17)	52.9 (20.5)	46.8 (17.0)	- 6.2 (- 18.0-5.6)	p=0.284
p-value [b]	p=0.376	p=0.445		
		Emotional wellbeing		
MFD (n=22)	49.1 (28.5)	63.3 (18.6)	+ 14.2 (0.0-28.4)	p=0.050
CFD (n=16)	61.3 (22.9)	64.5 (15.5)	+ 3.3 (- 6.0-12.5)	p=0.464
PFD (n=37)	57.1 (22.3)	69.3 (16.4)	+ 12.2 (5.1-19.3)	p=0.001

Table B. Quality of life scores of Short Form 36 across FD subtypes* (*continued*)

Subgroup	Baseline mean (±SD)	Follow up mean (±SD)	Difference (95% CI)	p-value [a]
MAS (n=17)	50.4 (29.3)	70.8 (10.9)	+ 20.5 (4.1-36.8)	p=0.017
p-value [b]	p=0.401	p=0.355		
Role limitations due to emotional problems				
MFD (n=22)	66.7 (38.5)	66.7 (39.8)	0.0 (- 21.9-21.9)	p=1.000
CFD (n=16)	66.7 (47.1)	83.3 (32.2)	+ 16.7 (- 8.5-41.8)	p=0.178
PFD (n=37)	70.3 (36.7)	81.1 (37.3)	+ 10.8 (- 0.9-22.5)	p=0.070
MAS (n=17)	78.4 (37.2)	76.5 (38.7)	- 2.0 (- 21.6-17.6)	p=0.835
p-value [b]	p=0.787	p=0.464		
Social Functioning				
MFD (n=22)	65.3 (23.8)	74.4 (23.0)	+ 9.1 (0.0-18.2)	p=0.050
CFD (n=16)	66.4 (26.9)	81.3 (20.4)	+ 14.8 (2.9-26.8)	p=0.018
PFD (n=37)	74.0 (24.9)	80.1 (21.3)	+ 6.1 (- 1.6-13.7)	p=0.116
MAS (n=17)	77.9 (20.5)	69.9 (21.2)	- 8.1 (- 17.4-1.3)	p=0.085
p-value [b]	p=0.301	p=0.321		
General Health				
MFD (n=22)	52.3 (26.9)	52.3 (22.7)	0 (- 6.0-6.0)	p=1.000
CFD (n=16)	53.8 (21.3)	57.8 (17.7)	+ 4.1 (- 3.1-11.2)	p=0.245
PFD (n=37)	59.7 (17.6)	59.5 (18.2)	- 0.3 (- 4.7-4.2)	p=0.903
MAS (n=17)	53.8 (19.2)	52.1 (19.9)	- 1.8 (- 10.4-6.9)	p=0.670
p-value [b]	p=0.534	p=0.433		
Health change compared to 1 year ago				
MFD (n=22)	40.9 (26.2)	46.6 (22.2)	+ 5.7 (- 9.2-20.6)	p=0.436
CFD (n=16)	46.9 (12.5)	56.3 (21.4)	+ 3.4 (- 3.4-22.1)	p=0.138
PFD (n=37)	46.6 (20.5)	49.3 (19.1)	+ 2.7 (- 6.4-11.9)	p=0.554
MAS (n=17)	47.1 (23.2)	44.1 (20.8)	- 2.9 (- 20.5-14.6)	p=0.727
p-value [b]	p=0.739	p=0.360		

*: Higher scores reflect better QoL. Range: 0-100. a: Paired samples t-test, b: One way ANOVA

Table C. Pain scores of BPI in patients with moderate-severe pain for FD-subtypes *

Subgroup	Baseline median (Q1-Q3)	Follow up median (Q1-Q3)	Difference median (Q1-Q3)	p-value [a]
		Maximum pain		
MFD (n=14)	7.5 (4.75-8)	6 (4-7.25)	- 0.5 (- 2.25-0.25)	p=0.101
CFD (n=10)	6.5 (4.75-7.25)	5.5 (2-7)	- 1.5 (- 3-0.25)	p=0.048
PFD (n=27)	7 (4-8)	7 (5-8)	0 (- 2-2)	p=0.955
MAS (n=11)	7 (5-8)	7 (6-8)	0 (- 2-1)	p=0.764
p-value [b]	p=0.842	p=0.442		
		Average pain		
MFD (n=14)	5 (3.25-7)	4.5 (2.75-7)	- 0.5 (- 2-0)	p=0.232
CFD (n=10)	3 (3-4.25)	3 (2-4)	- 0.5 (- 1.5-1)	p=0.389
PFD (n=27)	4 (3-7)	5 (2-6)	0 (- 2-1)	p=0.145
MAS (n=11)	5 (3-6)	4 (3-6)	0 (- 2-1)	p=0.256
p-value [b]	p=0.165	p=0.175		
		Interference of pain with general activity		
MFD (n=14)	7 (4-8)	6 (1.75-8)	- 0.5 (- 3.25-1.25)	p=0.227
CFD (n=10)	4 (1.75-7.25)	1 (0-2)	- 2 (- 5.25—0.75)	p=0.012
PFD (n=27)	5 (1-6)	4 (0-6)	0 (- 2-1)	p=0.357
MAS (n=11)	5 (2-8)	5 (3-7)	1 (- 2-2)	p=1.00
p-value [b]	p=0.138	p=0.017		

*: in patients with maximum or average pain at baseline ≥4. Higher scores=more pain and more interference. Range 0-10. a: Wilcoxon signed rank test, comparison between time points, b: Kruskal Wallis test

Table D. Pain scores of BPI in patients with pain score >0 for FD subtypes *

Subgroup	Baseline median (Q1-Q3)	Follow up median (Q1-Q3)	Difference median (Q1-Q3)	p-value [a]
		Maximum pain		
MFD (n=21)	5 (2.5-8)	5 (2.5-7)	-1 (- 2-1)	p=0.364
CFD (n=14)	5.5 (2-7)	5 (1.75-6.25)	-0.5 (- 2.25-1)	p=0.323
PFD (n=33)	7 (3-7.5)	6 (3.5-8)	0 (- 1-1.5)	p=0.782
MAS (n=16)	5 (1.25-7.75)	6 (3.25-7)	0 (- 1-2)	p=0.438
p-value [b]	p=0.841	p=0.405		
		Average pain		
MFD (n=21)	4 (1.5-6.5)	4 (2-6.5)	0 (- 1-0)	p=0.906
CFD (n=14)	3 (2-4)	2.5 (1.75-3.25)	-0.5 (- 1-1)	p=0.330
PFD (n=33)	4 (3-6)	4 (2-6)	0 (- 1-1)	p=0.371
MAS (n=16)	3.5 (1.25-5.75)	3 (2-4.75)	0 (- 1.5-1)	p=0.569
p-value [b]	p=0.460	p=0.184		

Table D. Pain scores of BPI in patients with pain score >0 for FD subtypes * (*continued*)

Subgroup	Baseline median (Q1-Q3)	Follow up median (Q1-Q3)	Difference median (Q1-Q3)	p-value [a]
Interference of pain with general activity				
MFD (n=21)	4 (0-7)	5 (0-6.5)	0 (- 2-2)	p=0.825
CFD (n=14)	2.5 (0-5.5)	1 (0-2)	-1 (- 4.25-0)	p=0.036
PFD (n=33)	4 (1-6)	3 (1-6)	0 (- 2-1)	p=0.232
MAS (n=16)	2.5 (1.25-6.75)	3 (2-5.75)	1 (- 1.75-1.75)	p=0.863
p-value [b]	p=0.768	p=0.042		

*: in patients with pain at baseline, maximum or average pain >0. Higher scores=more pain and more interference. Range 0-10. a: Wilcoxon signed rank test, comparison between time points, b: Kruskal Wallis test

Table E. Change in SF-36 scores for treatment groups during follow-up *

Subgroup	Baseline mean (±SD)	Follow up mean (±SD)	Difference (95% CI)	p-value [a]
SF-36 physical function				
FD-related therapy (n=50)	64.7 (24.1)	70.7 (23.8)	+ 6.0 (1-7-10.3)	p=0.007
No therapy (n=42)	76.7 (23.2)	78.7 (20.4)	+ 2.0 (- 2.3-6.4)	p=0.355
p-value [b]	p=0.018	p=0.091		
SF-36 role physical				
FD-related therapy (n=50)	44.0 (44.8)	52.5 (45.2)	+ 8.5 (- 5.0-22.0)	p=0.212
No therapy (n=42)	53.6 (44.0)	74.4 (38.1)	+ 20.8 (7.2-34.5)	p=0.004
p-value [b]	p=0.306	p=0.013		
Emotional wellbeing				
FD-related therapy (n=50)	50.6 (26.5)	69.0 (17.4)	+ 18.5 (10.5-26.4)	p<0.001
No therapy (n=42)	59.5 (23.3)	65.2 (14.1)	+ 5.7 (- 1.0-12.4)	p=0.092
p-value [b]	p=0.091	p=0.259		
Social functioning				
FD-related therapy (n=50)	68.5 (24.1)	75.8 (21.6)	+ 7.3 (0.6-13.9)	p=0.033
No therapy (n=42)	74.7 (24.5)	78.6 (21.8)	+ 3.9 (-2.8-10.5)	p=0.246
p-value [b]	p=0.225	p=0.536		

*: FD-related therapy: antiresorptive therapy or surgery
a: paired samples t-test, parametric comparison between time points
b: independent samples t-test, parametric comparison between groups

Table F. Change in pain scores for treatment groups during follow-up *

Subgroup	Baseline median (Q1-Q3)	Follow up median (Q1-Q3)	Difference median (Q1-Q3)	p-value [a]
		Maximum pain		
FD-related therapy (n=46)	7 (4-8)	6 (4-8)	0 (- 2-1)	p=0.587
No therapy (n=38)	4 (2-7)	5 (2-7)	0 (- 2-2)	p=0.967
p-value [b]	p=0.021	p=0.035		
		Average pain		
FD-related therapy (n=46)	4 (3-6)	3 (2-6)	0 (- 2-1)	p=0.089
No therapy (n=38)	3 (1-4.25)	3 (1-5)	0 (- 1-1)	p=0.912
p-value [b]	p=0.014	p=0.0147		
		Pain interference		
FD-related therapy (n=46)	5 (2-7)	3 (1-6.25)	0 (- 3-1)	p=0.061
No therapy (n=38)	2 (0-4.25)	2 (0-5)	0 (- 1-1)	p=0.686
p-value [b]	p=0.002	p=0.063		

*: FD-related therapy: antiresorptive therapy or surgery, in patients with pain score >0
a: Wilcoxon signed rank test, non-parametric comparison between time points
b: Mann Whitney U test, non-parametric comparison between groups

Table G. Illness perceptions *

Subgroup	Baseline mean (±SD)	Follow up mean (±SD)	p-value [a]
		Timeline acute/chronic	
Total (n=86)	18.7 (1.8)	18.8 (1.6)	p=0.574
New (n=25)	18.6 (1.7)	18.5 (2.1)	p=0.937
Chronic (n=61)	18.7 (1.9)	18.9 (1.4)	p=0.455
p-value [b]	p=0.766	p=0.323	
MFD (n=20)	18.8 (2.0)	18.8 (1.7)	p=1.000
CFD (n=15)	18.8 (1.4)	19.3 (1.2)	p=0.192
PFD (n=35)	18.5 (2.1)	18.7 (1.8)	p=0.811
MAS (n=16)	18.6 (1.2)	18.6 (1.5)0	p=1.000
p-value [c]	p=0.963	p=0.557	
		Timeline cyclical	
Total (n=86)	12.1 (3.9)	12.3 (4.1)	p=0.629
New (n=25)	11.2 (3.3)	11.4 (4.3)	p=0.839
Chronic (n=61)	12.5 (4.1)	12.7 (3.9)	p=0.656
p-value [b]	p=0.179	p=0.183	
MFD (n=20)	11.5 (3.5)	10.5 (4.3)	p=0.311
CFD (n=15)	10.6 (3.9)	11.2 (4.5)	p=0.580
PFD (n=35)	12.6 (3.9)	13.2 (3.6)	p=0.279
MAS (n=16)	13.3 (4.3)	13.7 (3.6)	p=0.562
p-value [c]	p=0.189	p=0.032	

Table G. Illness perceptions * (*continued*)

Subgroup	Baseline mean (±SD)	Follow up mean (±SD)	p-value [a]
	Consequences		
Total (n=86)	15.4 (3.5)	14.9 (3.6)	p=0.047
New (n=25)	15.3 (3.3)	14.4 (3.1)	p=0.114
Chronic (n=61)	15.5 (3.6)	15.1 (3.7)	p=0.205
p-value [b]	p=0.830	p=0.439	
MFD (n=20)	15.0 (3.5)	14.1 (3.4)	p=0.036
CFD (n=15)	13.8 (2.5)	13.4 (2.3)	p=0.546
PFD (n=35)	15.5 (3.1)	15.2 (3.3)	p=0.521
MAS (n=16)	17.3 (4.3)	16.8 (4.5)	p=0.333
p-value [c]	p=0.043	p=0.035	
	Personal control		
Total (n=86)	16.7 (2.5)	16.5 (2.2)	p=0.542
New (n=25)	16.2 (2.7)	16.2 (2.2)	p=1.000
Chronic (n=61)	16.9 (2.4)	16.6 (2.2)	p=0.427
p-value [b]	p=0.268	p=0.412	
MFD (n=20)	17.7 (2.1)	17.4 (2.1)	p=0.635
CFD (n=15)	15.2 (2.9)	15.5 (2.2)	p=0.691
PFD (n=35)	16.7 (2.3)	16.4 (1.6)	p=0.487
MAS (n=16)	16.8 (2.5)	16.6 (2.9)	p=0.835
p-value [c]	p=0.034	p=0.066	
	Treatment control		
Total (n=86)	15.4 (2.3)	15.8 (2.2)	p=0.204
New (n=25)	15.2 (2.6)	15.6 (2.4)	p=0.507
Chronic (n=61)	15.5 (2.2)	15.9 (2.1)	p=0.281
p-value [b]	p=0.583	p=0.636	
MFD (n=20)	15.7 (1.6)	15.9 (1.7)	p=0.778
CFD (n=15)	15.5 (1.8)	15.9 (2.8)	p=0.565
PFD (n=35)	15.0 (2.4)	15.7 (2.4)	p=0.113
MAS (n=16)	16.0 (3.3)	16.0 (1.7)	p=1.000
p-value [c]	p=0.472	p=0.952	
	Illness coherence		
Total (n=86)	14.0 (2.0)	14.1 (1.8)	p=0.614
New (n=25)	13.8 (1.9)	14.6 (2.0)	p=0.142
Chronic (n=61)	14.1 (2.0)	13.9 (1.8)	p=0.608
p-value [b]	p=0.639	p=0.156	
MFD (n=20)	14.3 (2.0)	14.4 (1.5)	p=0.748
CFD (n=15)	13.4 (1.8)	14.4 (2.5)	p=0.221
PFD (n=35)	14.1 (2.2)	13.9 (1.7)	p=0.746
MAS (n=16)	14.1 (1.9)	13.9 (1.9)	p=0.714
p-value [c]	p=0.615	p=0.712	

Table G. Illness perceptions * (*continued*)

Subgroup	Baseline mean (±SD)	Follow up mean (±SD)	p-value [a]
	Emotional response		
Total (n=86)	13.4 (3.5)	12.6 (4.1)	p=0.042
New (n=25)	14.0 (3.6)	13.7 (4.6)	p=0.726
Chronic (n=61)	13.2 (3.4)	12.2 (3.9)	p=0.024
p-value [b]	p=0.346	p=0.130	
MFD (n=20)	14.5 (3.9)	13.3 (3.9)	p=0.091
CFD (n=15)	14.0 (3.3)	11.1 (3.7)	p=0.005
PFD (n=35)	13.2 (3.5)	13.0 (4.3)	p=0.711
MAS (n=16)	12.0 (2.9)	12.6 (4.3)	p=0.488
p-value [c]	p=0.171	p=0.378	

* Range of scores: 6-30 for timeline acute/chronic, consequences, personal control and emotional representation, 4-20 for timeline cyclical, 5-25 for treatment control and illness coherence. High scores for personal/treatment control and illness coherence indicate positive beliefs about controllability and understanding of the disease. High scores on timeline acute/chronic/cyclical, consequences and emotional response represent strong beliefs on the acute/chronic/cyclical nature and on negative consequences and emotions caused by the disease. a: paired samples t-test; comparison between time points, b: independent samples t-test; comparison between hospitals, c: one way ANOVA; comparison between diagnosis types

Table H. Missing data analysis - comparison of characteristics of all patients included > 1 year in PROFID study

Characteristics	Completers of both surveys N=92	Dropout after 1st survey N=26	No surveys completed N=6	p-value
Age at baseline (mean, ±SD)	44.8 (15.2)	34.9 (15.8)	41.5 (19.4)	0.018 [a]
Sex, female (number, %)	61 (66.3%)	20 (76.9%)	3 (50%)	0.344 [b]
Type of FD (number, %)				0.657 [b]
MFD non CFD	22 (23.9%)	6 (23.1%)	1 (16.7%)	
Isolated CFD	16 (17.4%)	7 (26.9%)	2 (33.3%)	
PFD	37 (40.2%)	7 (26.9%)	3 (50%)	
MAS	17 (18.5%)	6 (23.1%)	0	
Skeletal Burden Score (median, Q1-Q3)	3.2 (1.2-16.9)	2.8 (1.3-17.7)	7.8 (2.5-18.8)	0.806 [c]
Baseline QoL (mean, SD)				
Physical function	70.2 (24.3)	69.6 (26.5)	N/A	0.921 [d]
Vitality	50.9 (20.1)	48.8 (18.9)		0.647 [d]
General Health	55.8 (21.0)	52.5 (21.0)		0.478 [d]
Baseline average pain score (median, Q1-Q3)	3 (2-5)	4 (2-5)	N/A	0.824 [d]

a: one-way ANOVA, b: Chi-square test, c: Kruskall Wallis test, d: Mann-Whitney U test

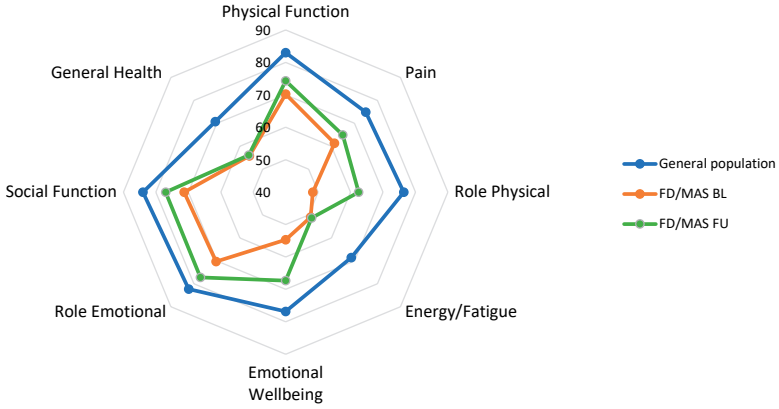

Figure A. SF-36 at baseline and follow-up in patients with FD/MAS versus general Dutch population

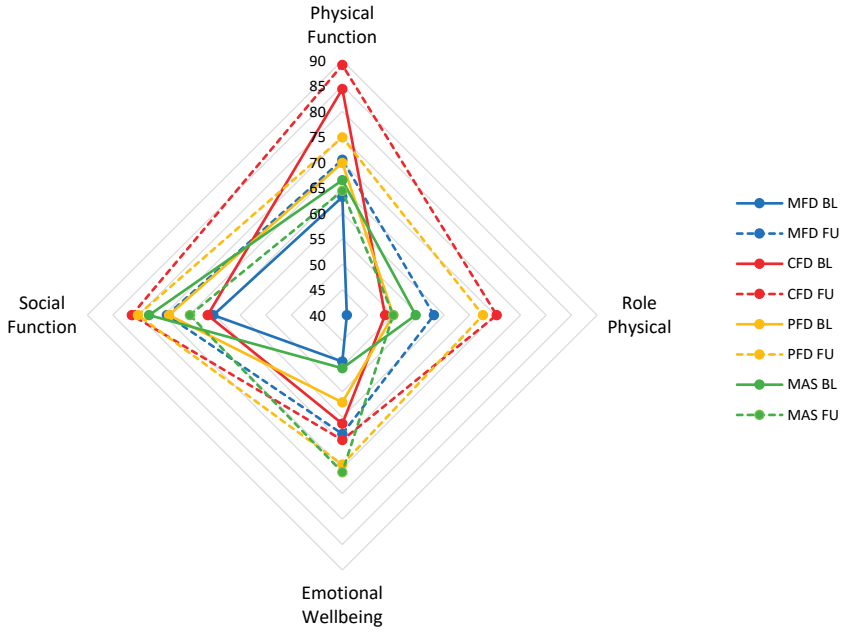

Figure B. SF-36 domains at baseline and follow-up for FD-subtypes

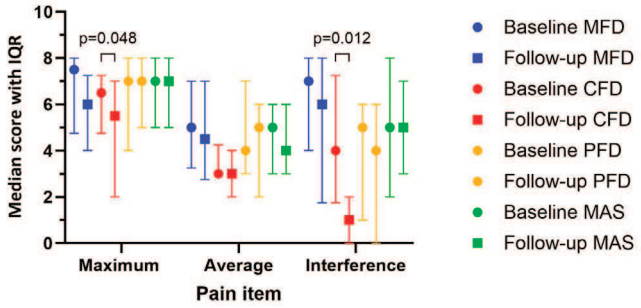

Figure C. Pain scores in patients with moderate to severe pain over time across FD subtypes

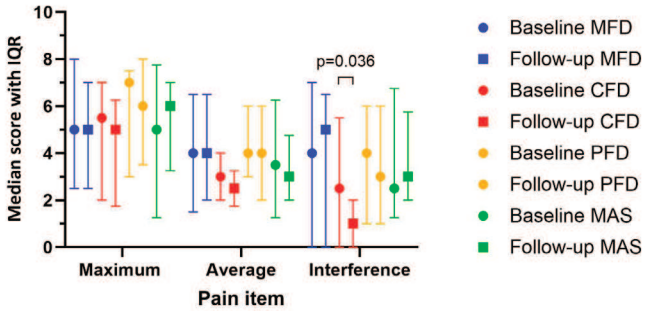

Figure D. Pain scores in patients with pain score > 0 over time across FD subtypes

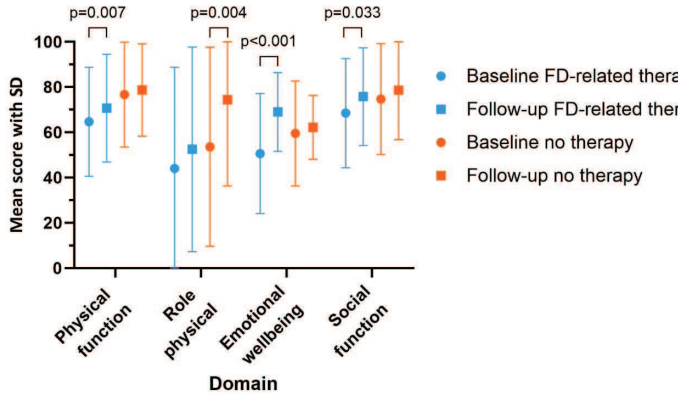

Figure E. SF-36 scores at baseline and follow-up across treatment during follow-up

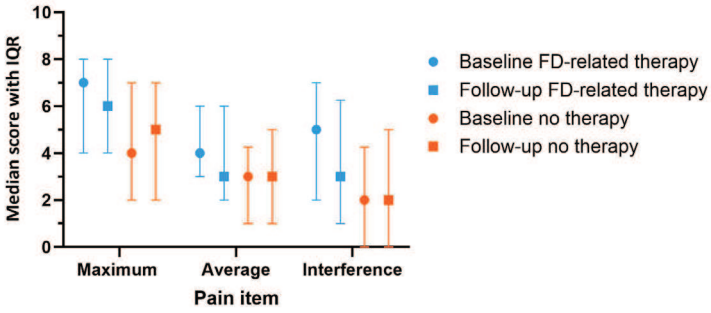

Figure F. Pain scores at baseline and follow-up across treatment during follow-up

Chapter 8

Safety of Therapy with and Withdrawal from Denosumab in Fibrous Dysplasia and McCune-Albright Syndrome: an observational study

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Abstract

Denosumab treatment can benefit patients with fibrous dysplasia/McCune-Albright Syndrome (FD/MAS) by suppressing the RANKL-mediated increased bone resorption. However, limited data of two pediatric cases indicate that a rebound phenomenon may occur after withdrawal. Therefore we studied the safety of denosumab discontinuation in FD/MAS. Thirty-seven patients using denosumab, mostly after unsuccessful bisphosphonate treatment, were included. Health records were screened for pain scores, side effects and bone turnover markers (BTMs) (calcium, alkaline phosphatase (ALP), procollagen 1 N-terminal propeptide (P1NP) and β-crosslaps (CTX)) during treatment, and for BTMs and clinical rebound effects after withdrawal. BTM levels after withdrawal were compared to pretreatment values. Data were calculated as median(IQR). BTMs normalized in 2/3 of patients and pain scores decreased significantly during treatment (p=0.002). 1 patient (2.7%) developed osteonecrosis of the jaw. Sixteen patients discontinued denosumab treatment after a median of 1.6(IQR 1.0) years because of insufficient effect on pain (n=10,63%), side effects (n=4,25%) or other (n=4,25%). Follow-up post-treatment was 3.2(2.8) years, wherein no fractures, pain flares or lesion progression occurred. Calcium remained normal in all but 1 patient, who had a mild asymptomatic hypercalcemia (2.73 mmol/L) 5 months after discontinuation. ALP passed pretreatment levels in 5/11 patients (46%), increased most after 6 months by 18(43) U/L and returned to baseline levels thereafter. P1NP exceeded pretreatment levels in 4/9 patients (44%), CTX in 8/9 (89%). P1NP rose most after 3 months and stabilized thereafter. CTX showed the highest relative elevation. Patients with high pretreatment levels responding well to denosumab seemed to have the highest rebound. These results suggest beneficial effects of denosumab on pain and BTMs, and show a biochemical but asymptomatic rebound phenomenon after withdrawal in adults with FD/MAS, mainly in case of high pretreatment levels, good response and multiple injections. Further studies on the safety of denosumab and withdrawal are needed and ongoing.

Introduction

Fibrous dysplasia (FD) is a rare genetic disorder characterized by the presence of fibrous skeletal lesions in one or multiple bones (monostotic vs polyostotic, MFD vs PFD), sometimes accompanied by intramuscular myxomas in the Mazabraud syndrome (MZB) or hyperfunctioning endocrinopathies in the McCune-Albright syndrome (MAS)[1-3]. FD/MAS is induced by a postzygotic mutation in the *GNAS* gene, encoding the α-subunit of the stimulatory G-protein (Gsα). Altering various downstream cascades, the mutation disturbs bone remodeling and endocrine functions in MAS[4]. In bone, the mutation occurs in osteoprogenitor cells (OPC) and hampers their differentiation into mature osteoblasts. In addition, the mutated OPCs stimulate osteoclast survival and activity through increased levels of cytokines, such as Interleukin 6 (IL-6) and Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL). RANKL is a member of the tumor necrosis factor family and has been detected excessively in both FD bone tissue and serum of patients. RANKL also has been shown to correlate with skeletal burden in FD/MAS[5-8]. The above processes lead to an increase in both bone formation and bone resorption and to the development of weak, immature, woven bone lesions with disturbed adipogenesis and hematopoiesis. These lesions can induce a wide spectrum of symptoms including deformities, pain, and fractures[9, 10]. For symptomatic patients, mechanic or medical treatment is necessary and patients require pain medication, surgery or antiresorptive drugs, mainly bisphosphonates [10, 11]. However data on the effect of bisphosphonates are scattered with oral preparations not being effective in treating bone pain[12], and intravenous bisphosphonates showing reductions in pain and markers of bone turnover in many patients, but not all[13-17]. The underlying overproduction of RANKL has led to the use of denosumab (Dmab), a humanized monoclonal antibody, mimicking the function of the decoy receptor osteoprotegerin (OPG) by binding to RANKL and preventing the connection to its receptor RANK on the membrane of osteoclasts[18]. As a result, the formation and stimulation of these osteoclasts is inhibited, leading to a suppression of bone resorption and an elevation in bone mass [19, 20]. Denosumab is mainly used for the treatment of osteoporosis [19, 21, 22] and skeletal metastases[23, 24], and recently for giant cell tumors of bone[25, 26]. In a FD/MAS mouse model, Dmab halted the progression of lesions, induced the formation of mineralized bone within lesions and prevented the development of new FD lesions[27]. In humans successful treatment has been observed in bisphosphonate refractory patients, leading to diminished growth rate[28, 29] or even regression of lesions[30, 31], to pain reduction and to decreased bone turnover [32-35]. Nevertheless, data on the safety of long-term Dmab use and especially on Dmab withdrawal in FD/MAS have not been reported. Withdrawal from denosumab in osteoporosis patients can provoke rebound increases in

markers of bone turnover above pretreatment levels and enhances the risk for multiple vertebral fractures[36-38]. To prevent this, alternative antiresorptive post-treatment should be considered as it might prevent the rebound in the bone resorption markers, although its ability to prevent bone loss is still under debate[39, 40]. Besides bone loss in FD/MAS there is another concern as in the mouse model the lesions reoccurred after therapy cessation[27]. This was also observed in a case report of a young boy even resulting in such a severe rebound that besides rapid growth a life-threatening hypercalcemia occurred[28]. Also another child demonstrated a severe hypercalcemia after discontinuation from denosumab and lesional growth, although in a slower rate. As we recently reported the successful treatment of symptomatic FD/MAS with Dmab[35] we now set out to evaluate the safety of long-term use of Dmab in FD/MAS and to assess the effect of discontinuation on clinical parameters such as pain and fractures and on biochemical parameters such as serum calcium and markers of bone turnover.

Patients and methods

Patient selection

All patients with FD/MAS were included who were treated with denosumab injections and attending the outpatient clinics of the Center for Bone Quality of the Leiden University Medical Center (LUMC) and of the Department of Endocrinology of the Radboud University Medical Center (RUMC) (n=37). For the management of FD/MAS, a protocolled care trajectory is followed including regular assessments of pain, markers of bone turnover and radiological evaluation. Medical treatment involves suppletion of calcium and vitamin D3 or active D metabolites when necessary and symptomatic patients are, when a mechanic component is excluded, treated with analgesics and intravenous bisphosphonates. When these therapies are not tolerated or not (sufficiently) effective, which is for bisphosphonates defined as insufficient pain relief with biochemical progressive disease despite adequate treatment according to the guidelines[18], denosumab (Prolia®, Amgen) is offered after multidisciplinary consultation as last resort. Oral informed consent for the off-label use of denosumab is obtained from all patients. The present study is part of the PROFID-study, a multicenter observational cohort study for FD/MAS, in which data on clinical, biochemical, radiological and histological parameters are retrieved, as well as treatment effects and questionnaires on pain and quality of life. All patients have given informed consent prior to inclusion in the study. Approval from the Medical Ethics Committee of the LUMC was obtained.

Treatment protocol

A selection of the currently included patients was described before[35]. Currently, at our center Dmab is prescribed in a dose of 60 mg every 3 months[35]. Nine patients started on a 6-monthly schedule, 1 patients on a 4-monthly schedule and 21 patients on 3-monthly schedule. Follow up with clinical and biochemical evaluation including registration of adverse effects was scheduled every 3 months. Radiological examination is performed on indication. According to clinical and biochemical treatment response and side effects, the dosage or interval of denosumab treatment can be adjusted at the discretion of the treating physician, which was done in 12 patients. In 9 patients treatment was intensified because of insufficient effect on pain or bone turnover markers and in 4 patients tapered off because of satisfactory outcomes. After withdrawal, appointments are also scheduled every 3 months.

Data Collection

Patient data were collected in an online Castor database. Baseline characteristics were registered such as sex, type of FD, and skeletal burden score[41]. Clinical data concerning denosumab treatment were retrieved, such as age at start of treatment, treatment dosage and schedule and adverse effects. Pain was examined during treatment by the Brief Pain Inventory (BPI), assessing scores for maximum, minimum, mean and current pain on a scale from 0 to 10 as well as pain locations and the use of analgesics. In patients who discontinued treatment, clinical signs of rebound effects like pain flares, fractures or radiographic lesion progression were assessed at each visit during the entire follow up period after withdrawal. All serum laboratory measurements were collected of albumin-corrected calcium and the bone turnover markers alkaline phosphatase (ALP), procollagen type 1 N-terminal propeptide (P1NP) and collagen type 1 C-terminal telopeptide (b-crosslaps; B-CTX) performed for clinical purposes prior to starting denosumab, during treatment, and after withdrawal in a non-fasting state. Levels of albumin-corrected calcium (calcium reference range is 2.15-2.55 mmol/L) and phosphate (0.90-1.50 mmol/L) were measured by semi-automated techniques. ALP was determined by an automated P800 modulator system (Roche BV, Woerden, The Netherlands), the upper limit of normal (ULN) ALP <98 IU/L for women and <115 for men. P1NP, ULN <59 ng/mL for premenopausal women and men, and <76 ng/ml for postmenopausal women, and B-CTX, ULN <0.573 for premenopausal and <1.008 ng/mL for postmenopausal women, <0.704 for men until 70 years and 0.854 for men >70 years old, were measured by the E-170 system (Roche BV, Woerden, The Netherlands). The C-terminal of FGF-23 (Immutopics, San Clemente, CA, USA), ULN <125 RU/ml, was measured by the BioTek ELx50 Washer (BioTek, Bad Friedrichshall, Germany). All analyses were performed according to the manufacturers' protocol. To evaluate

treatment response, the values of calcium and the BTMs closest to 12 months after the start of treatment (median: 12, IQR: 11-13, range: 10-20 months) were compared with baseline pretreatment levels. Similarly the values of calcium and BTMs after discontinuation were grouped, while all measurements performed between two time points were regarded as measured at the first time point, at 3 months (median 5, range 4-5), 6 months (median 7.5, range 6-9) and 12 months (median 13, range 12-21) after the last injection. These values were compared to the last available measurement before or within 3 months after the last injection.

Statistical analyses

Statistical analyses were performed in SPSS Statistics for Windows, version 25.0 (SPSS Inc., Chicago, IL.) after data extraction from Castor. Results are described as number and percentage for categorical data and as median (interquartile range) for numerical data unless stated otherwise. The courses of scores for maximum and average pain during treatment were analyzed in a linear mixed model. Lab markers values were described as absolute values, absolute change and relative change. Laboratory marker values after 1 year of treatment were compared with pretreatment values in a Wilcoxon signed rank test in case of symmetrical distribution of differences and in a sign test in the remainder of the comparisons. For lab marker values after withdrawal the number of patients was calculated in whom laboratory markers after withdrawal exceeded pretreatment levels and exceeded the individual upper reference limit at any time during the first 18 months after the last injection. Significance level was set at $p<0.05$. Spaghetti graphs were computed to depict the percentage change in calcium and BTMs after withdrawal. Graphs were designed in Graphpad Prism, version 8.4.2 (GraphPad Software, La Jolla California).

Results

Patients' characteristics

Thirty-seven patients (table 1) were treated with denosumab (24 females (64.9%), of whom 8 postmenopausal). Seven patients had MFD (18.9%), but the majority of patients had polyostotic FD (n=21, 56.8%). Nine patients were diagnosed with MAS (24.3%). The median SBS was 11.8 (IQR 23.6).

34 patients (91.9%) were previously treated with bisphosphonate therapy, with a median cumulative dose of 713 (range: 0-6336) mg over 6.0 (range: 0-22.1) years. This treatment was in all cases unsatisfactory. Three patients had not used bisphosphonates. One of them was diagnosed with osteoporosis, for which denosumab treatment was initiated elsewhere, and 2 patients had FD in the thoracic region, 1 with MFD and 1 with MAS, and preferred Dmab over BP therapy due to fear for side effects. Six patients had received a single administration whereas 31 patients were treated with multiple injections over a median duration of 3.5 years (IQR 3.0, range 0.5-5.3), with a median of 11 injections (IQR 13, range 2-20) in a median cumulative dose of 660 mg (IQR 900, range 120-1860). Reasons for withdrawal are discussed below in section 'Outcomes of withdrawal'.

Table 1. Baseline characteristics

Number of patients	**n = 37 (% or IQR)**	
Gender		
Female	24	(64.9 %)
Male	13	(35.1%)
Age at start denosumab therapy	42	(19)
Type FD		
Monostotic	7	(18.9 %)
Polyostotic	21	(56.8 %)
MAS	9	(24.3 %)
Skeletal Burden Score	11.8	(23.6)
Bone turnover markers prior to denosumab therapy		
Corrected calcium (n = 37) Normal: 2.15-2.55 mmol/l	2.31	(0.13)
Phosphate (n = 36) Normal: 0.90-1.50 mmol/l	0.96	(0.25)
ALP (n = 37) Normal: women: <98, men: <115 U/l	103	(127)
P1NP (n = 33) Normal: men & premenop. women: <59 ng/ml Postmenop. women: <76 ng/ml	85	(259)
B-CTX (n = 33) Normal: women, premenop.: <0.573, postmenop.: <1.008 ng/ml Men, ≤70 y: <0.704, >70 y: <0.854 ng/ml	0.306	(0.245)
FGF-23 (n = 25) Normal: <125 RU/ml	132	(84)
Bisphosphonate therapy		
BP use prior to Denosumab	34	(91.9 %)
Duration, y (range)	5.3	(0-22.1)

Data are presented as number and percentage or as median and IQR

Table 2. Course of bone turnover markers before treatment, during treatment and after withdrawal

N = 11	**Last before treatment**	**After 1 year of treatment**		**6 months after withdrawal**		**12 months after withdrawal**	
BTM	Value	Value	Change *	Value	Change +	Value	Change+
Ca, mmol/L	2.31 (0.13)	2.37 (0.17)	- 0.01 (0.22)	2.36 (0.17)	+ 0.2 (0.43)	2.33 (0.12)	+ 0.01 (0.20)
ALP, U/L	103 (127)	83 (52)	- 35 (83)	94 (170)	+ 18 (43)	86 (127)	+ 10 (66)
P1NP, ng/mL	85 (259)	36 (153)	- 45 (85)	62 (401)	+ 40 (132)	81 (56)	+ 35 (50)
B-CTX, ng/mL	0.31 (0.25)	0.13 (0.25)	- 0.12 (0.37)	0.34 (0.86)	+ 0.17 (0.42)	0.34 (0.40)	+ 0.27 (0.41)

Data are presented as median (IQR). * Compared to last before treatment, + compared to last before withdrawal.

Normal ranges: Ca 2.15-2.55 mmol/l, ALP women: <98, men: <115 U/l, P1NP: men & premenopausal women: <59 ng/ml, postmenopausal women: <76 ng/ml, CTX: women, premenopausal: <0.573, postmenopausal: <1.008 ng/ml, men ≤70 y: <0.704, men >70 y: <0.854 ng/ml

Treatment outcomes

Laboratory markers

Median ALP at baseline was 103 U/L (IQR 127) and was elevated in 20/37 patients (54.1%). ALP normalized in 14/20 patients (70%) and decreased to 83 (IQR 52) U/L after 1 year of treatment (sign test p=0.001) (table 2, figure 1). P1NP and B-CTX were available at baseline in 33 patients and in 29 patients also during treatment. At baseline, P1NP was elevated in 20/33 patients (60.1%), and normal values were reached in 14 of them (70%) while on treatment. After 1 year of treatment P1NP diminished from 85 (IQR 259) ng/ml to 36 (153) ng/ml (sign test p=0.005). B-CTX before treatment exceeded the ULN in 4/33 patients (11.4%) and normalized in 3 of them (75%) during treatment. B-CTX declined from 0.31 (IQR 0.27) ng/ml at baseline to 0.13 (0.25) ng/ml after 1 year (Wilcoxon signed rank test p=0.044) (table 2, figure 1).

Pain and use of analgesics

Pain scores fluctuated in many patients during treatment. Scores during the first 2 years of treatment were included in the linear mixed model analysis because of a diminishing number of measurements thereafter. During this time frame scores for maximum pain decreased significantly from a mean of 6.0/10 (SD 2.7) at baseline by β=0.09 per month (95%CI: 0.03-0.15, p=0.002). In other words, after 11 months of treatment maximum pain scores declined on average by 1 point (95%CI: 0.4-1.6), and after 2 years on average by 2.7 points (95%CI: 0.8-3.6). Scores for average pain decreased significantly from a mean of 4.4 (SD 2.4) at baseline by β=0.05 per month (95%CI: 0.005-0.099, p=0.48), indicating a decline of 1.2 points (95%CI: 0.01-2.2) after 2 years of treatment. Analgesics

were used by 18 patients (48.6%) at the start of denosumab therapy. Fourteen patients (37.8%) did not use any analgesics and data were missing in 5 patients (13.5%). Of those 18 patients, 6 were taking opioids. Four of them continued the use of opiates during denosumab therapy (66.7%), 1 could decrease the dosage of the opioids (16.7%), and 1 patient (16.7%) could withdraw completely from all painkillers during denosumab therapy. Of the remaining 12 patients using acetaminophen and/or NSAIDs prior to denosumab, 3 (25%) could reduce the use of pain killers, 7 (58.3%) continued the medication and 2 patients (16.7%) used analgesics infrequently. Of the 14 patients not using analgesics before the first denosumab injection, 4 (28.6%) used acetaminophen or NSAIDs infrequently and 10 (71.4%) used no analgesics during denosumab treatment. Four patients specifically reported that denosumab therapy had increased their quality of life despite unchanged pain levels in 3 of them. This occurred because these patients felt less tired and more active or because more (physical) activity was possible.

Side effects

Twenty patients (51.4%) reported in total 37 adverse effects. Side effects were severe in 2 patients: 1 patient (2.7%) reported oral blisters and 1 patient (2.7%) developed osteonecrosis of the jaw (ONJ). The latter was a 35-year-old male who was diagnosed with MAS with a SBS of 31.40 and had received 360 mg of denosumab in 1.5 year. He had also been treated with bisphosphonates for 12 years, but this was stopped 8 years before the start of denosumab treatment. The ONJ developed after a dental procedure without antibiotics prophylaxis nor primary suture of the lesion. In both patients denosumab therapy was discontinued because of these side effects. The majority of the side effects were mild to moderate, specifically malaise (n=7, 18.9%), skin problems, mainly rash or dryness (n=6, 16.2%), muscle pain of stiffness (n=5, 13.5%), flare in FD related pain (n=5, 13.5%), which was in all cases self-limiting, diarrhea and flatulence (n=3, 8.1%), fatigue (n=2, 5.4%), tooth sensitivity (n=2, 5.4%), nausea and vomiting (n=1, 2.7%), headache (n=1, 2.7%), brittle nails (n=1, 2.7%), recurrent respiratory tract infections (n=1, 2.7%), and the sensation of bladder pressure (n=1, 2.7%) (table 3).

Outcomes of withdrawal from denosumab

Clinical parameters

In 16 of 37 patients (43%) the use of denosumab therapy was ceased (table 3). Two patients had MAS (14.3%), 4 had MFD (28.6%) and 8 PFD (57.2%). Six patients received a single injection and 10 patients were treated with multiple injections (median 4, IQR 6, range 3-11) in a median cumulative dose of 240 mg (IQR 390, range 120-780) during a median duration of 1.6 years (IQR 1.0, range 1.0-4.9). Reasons for withdrawal were (a combination of) insufficient relief of

Table 3. Adverse effects of denosumab therapy

Patients, total	N=20 (54.1% of total)
Adverse effects, total	N=37
Adverse effect	Patients, n (% of total)
Malaise	7 (18.9%)
Skin problems	6 (16.2%)
Muscle pain or stiffness	5 (13.5%)
Flare in FD related pain	5 (13.5%)
Diarrhea/flatulence	3 (8.1%)
Fatigue	2 (5.4%)
Tooth sensitivity	2 (5.4%)
Nausea/vomiting	1 (2.7%)
Headache	1 (2.7%)
Brittle nails	1 (2.7%)
Multiple respiratory tract infections	1 (2.7%)
Sensation of bladder pressure	1 (2.7%)
Vertigo	1 (2.7%)
Transient hyperparathyroidism	1 (2.7%)

pain (n=10, 62.5%) or side effects (n=4, 25%) being osteonecrosis of the jaw (n=1, 6.3%), oral blisters (n=1), rash (n=1) or both muscle ache and respiratory tract infections (n=1). Other reasons for withdrawal were family planning (n=1), complete pain alleviation after surgery (n=1), treated osteoporosis (n=1) and long-term use (n=1). The median clinical follow up after the last injection was 3.2 years (IQR 2.8). No patients experienced a flare in FD related pain after withdrawal. Moreover, no (atypical) fractures nor lesional growth were observed.

Laboratory marker values

Laboratory markers after withdrawal were available in 11 patients (table 2, figure 1), of whom 2 received a single injection and 8 had multiple injections. In 8/11 patients (72.7%) calcium levels exceeded pre-treatment levels within 18 months after withdrawal. However, in all but 1 patient (90.9%) calcium remained below the upper limit of normal. In 1 patient a mild, asymptomatic hypercalcemia of 2.73 mmol/L was observed 5 months after the last injection (figure 1C and 1D, patient 3). The patient was diagnosed with MAS and had used denosumab for 2.1 years (table; 4, patient 3). Treatment to decrease the calcium was not necessary. After 6 months the calcium was 2.45 mmol/ml, but denosumab had already been restarted because of gradually increasing FD-related pain. The median calcium level of the entire cohort increased slightly in the first 6 months after withdrawal but did not exceed the upper limit of normal (table 2).

Table 4. Characteristics of patients withdrawn from denosumab therapy

Patient ID	Age at start Dmab, gender	Type of FD/ MAS	Skeletal Burden Score	Duration of BP use (y) / cumulative dose (mg)	Duration of Dmab use (y) / cumulative dose (mg)	Dmab scheme	Follow up after last dose (y)
1	26, F	MFD	0.5	6.6 / 691	1.2 / 240	60 mg sc / 3 mo	1.6
2	59, F	PFD	2.8	10.0 / 75	1.6 / 180	60 mg sc / 6 mo	1.6
3	43, F	MAS	44.2	22.0 / 2204	2.1 / 540	60 mg sc / 3 mo	1.3
4	33, F	PFD	25.1	11.8 / 2314	2.7 / 780	60 mg sc / 6 mo > 60 mg / 4 mo > 120 mg / 3 mo	3.2
5	54, F	MFD	0.8	4.9 / 521	1.6 / 240	60 mg sc / 6 mo	2.5
6	28, F	PFD	13.8	8.6 / 4740	1.3 / 120	60 mg sc / 6 mo	3.6
7	42, F	PFD	0.4	2.5 / 3160	1.0 / 180	60 mg sc / 6 mo	4.1
8	33, M	MAS	31.4	11.8 / 3305	1.5 / 360	60 mg sc / 3 mo	3.2
9	38, M	PFD	43.3	0.01 / 0.60	1.3 / 240	60 mg sc / 6 mo > 60 mg / 3 mo > 60 mg / 6 mo	1.6
10	63, F	MFD	0.3	0 / 0	0 / 60	Single injection	4.8
11	58, F	PFD	0.1	2.2 / 735	0 / 60	Single injection	2.8
12	34, F	PFD	8.5	1.9 / 7	4.9 / 660	60 mg sc / 6 mo > 60 mg / 10 mo > 60 mg / 8 mo > 60 mg / 3 mo	0.4
13	39, M	PFD	15.8	3.0 / 1607	0 / 60	Single injection	4.7
14	42, F	PFD	17.1	4.8 / 20	0 / 60	Single injection	4.4
15	39, M	PFD	16.5	1.5 / 157	0 / 60	Single injection	4.1
16	63, M	MFD	0.4	0 / 0	0 / 60	Single injection	4.8

Abbreviations: F, female; M, male; FD, fibrous dysplasia; MAS, McCune-Albright syndrome; MFD, monostotic FD; PFD, polyostotic FD; Dmab, denosumab; BP, bisphosphonates, sc, subcutaneously.

In 9/11 patients (81.8%) at least one BTM exceeded pretreatment values and in 7 of them (77.8%), at least one of these BTMs also passed the ULN. In 2 patients (18.2%) all BTMs exceeded pretreatment levels, although in 1 patient ALP and B-CTX remained below the ULN (figure 1).

ALP exceeded pretreatment levels after withdrawal in 5/11 patients (45.5%), but remained below the ULN in 2. Median ALP levels peaked at 94 (170) U/L at 6 months, due to a median increase of 18 (IQR 43) U/L, and stabilized after 12 months. The highest increase in ALP was +25% compared to pretreatment values (table 2, figure 1A and 1B).

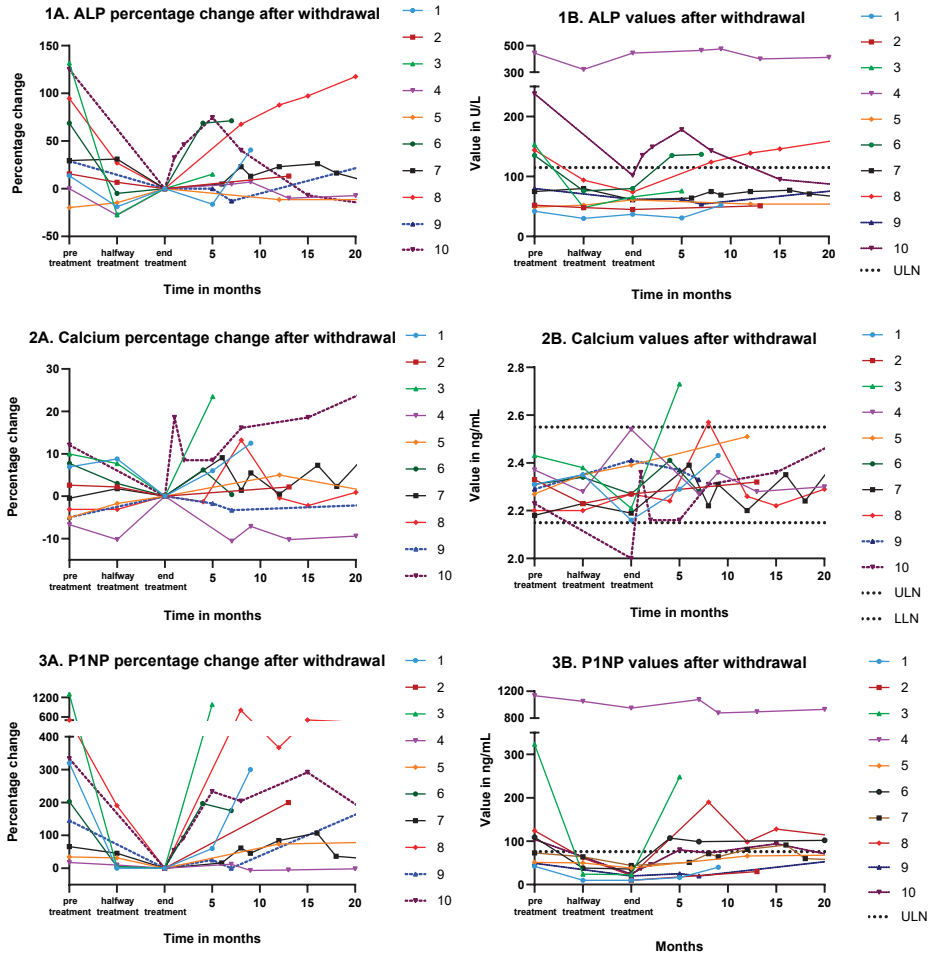

Figure 1A-F. Levels of calcium and of markers of bone turnovers ALP, P1NP and B-CTX during treatment and after withdrawal

Graphs demonstrating the values of albumin corrected calcium, ALP, B-CTX and P1NP before treatment, halfway during treatment, at the end of denosumab treatment and after withdrawal. The numbers below halfway and end treatment correspond to the median (interquartile range) and range. The dotted lines of patient 10 and 11 represent the patients who received a single injection. Calcium values remain generally stable and below the upper limit or normal (ULN), except for one patient. ALP, P1NP and B-CTX show a distinct increase after withdrawal in several patients, mainly in patients with high pretreatment levels. The steepest slope is observed in the first months after withdrawal. However in a subset of the patients the bone turnover markers remain quite stable.

P1NP and B-CTX levels after cessation were available in 10 patients, but in 1 patient pretreatment values were missing. In 4/9 (44.4%) patients P1NP exceeded pretreatment values, in 3 of them above the ULN. The greatest increase in P1NP was 153% compared to pretreatment. Median P1NP was 24 (IQR 22) ng/ml at

the end of treatment, rose to 80 (157) ng/ml 3 months after withdrawal and remained elevated around that level afterwards (table 2, figure 1E).

Of all lab markers, CTX showed the highest relative increase after denosumab discontinuation (figure 1). Median B-CTX during treatment was 0.067 (IQR 0.081) ng/ml and rose to 0.29 (IQR 0.18) after 6 months of withdrawal. In 8/9 patients (88.9%), B-CTX levels exceeded pretreatment levels, in 4 of them (50%) above the ULN. The maximum rise in B-CTX was +209% compared to pretreatment values. Overall, of all BTMs exceeding pretreatment values (n=17), median time to stabilization was 12.5 (IQR 17) months (table 2, figure 1F).

The laboratory markers calcium, ALP, B-CTX, and P1NP seem to increase and decrease simultaneously, because a few patients account for the elevations in all four laboratory marker values, whereas some other patients appear to remain stable in all values, as is depicted in figure 1. Mainly calcium, B-CTX, and P1NP follow identical curves. Patient 3 and 8 exhibit the highest rebound (figure 2). These patients were affected with MAS, had high pretreatment levels of BTMs and an excellent biochemical response to denosumab. They had used 2204 and 3305 mg bisphosphonates over 22 and 12 years respectively, and had used high doses of denosumab (540 and 360 mg) over 2.1 and 1.5 year. Patients with low rebound mainly had low levels before treatment. Those patients had MFD or PFD, but not MAS, and had used variable amounts of bisphosphonates and of denosumab. Remarkably, the patient with the highest pretreatment levels, the longest duration of denosumab use, and the highest cumulative dose showed a low rebound phenomenon. In this patient the levels of the BTMs remained high throughout the entire treatment. BTMs hardly increased after withdrawal: ALP rose from 444 to 475 U/L, P1NP from 1050 to 1177 ng/ml, B-CTX from 0.745 to 0.936 ng/ml, and calcium decreased after discontinuation. The two patients receiving a single injection of denosumab showed mild rebound effects. In 1 patient, all four lab markers remained within normal limits, although calcium, P1NP and B-CTX slightly exceeded pretreatment levels. The other patient had elevations in AF and P1NP to above the upper normal limit, although not above pretreatment values, and in calcium and B-CTX over pretreatment values but not over the ULN.

Antiresorptive therapy after withdrawal

Denosumab therapy had been restarted after withdrawal in 2 patients (12.5%). In both patients the most severe pain did not respond well to denosumab and was surgically treated. However, after a while the FD-related pain recurred in other lesions, for which denosumab was restarted successfully. Bisphosphonate therapy was never acutely indicated because of rebound phenomenon. One patient

had received zoledronic acid for a slow incline in P1NP levels. 6 patients were treated with bisphosphonate therapy because FD bone pain increased gradually over time, 1 patient received zoledronic acid according to the treatment protocol for osteoporosis, 1 patient received zoledronic acid twice after 5 years of Dmab therapy according to the treatment protocol for long-term use, and in 7 patients no further antiresorptive therapy was prescribed.

Discussion

The primary aim of this study was to evaluate the safety of withdrawal from denosumab treatment in patients with FD/MAS. Withdrawal from denosumab has not caused flares in FD related pain in our cohort, neither were fractures observed, nor progression of the lesions, although patients were under surveillance for a median of 3.2 years. However, the main concern regarding the discontinuation of denosumab is the rise in serum bone turnover markers and calcium, since two children have been reported who developed severe rebound effects in BTMs as well as hypercalcemia after discontinuation of therapy[28]. Hazardous hypercalcemia was not observed in our cohort and merely one patient developed a mild, asymptomatic hypercalcemia (serum calcium: 2.73 mmol/L) 5 months after the last injection. Next to hypercalcemia, this patient had a prominent increase in B-CTX, reflective of a very active bone resorption. An explanation for the discrepancy between our cohort and the two reported pediatric cases is that we only treated adults with matured skeletons of whom 92% were pretreated with bisphosphonates. Despite the absence of hypercalcemia, the majority of patients showed a relapse in bone turnover markers to around pretreatment levels and some to above pretreatment levels. Values after discontinuation became at maximum 25% higher than pretreatment for ALP, 143% for P1NP and 203% for B-CTX. Stabilization was observed after one year. In osteoporosis patients, BTMs also exceeded the pretreatment levels or the upper normal limit after discontinuation of denosumab, and rebound increases in bone turnover markers were observed 3-9 months after the last injection, although individual responses were variable just like in our study[38, 40]. In our cohort patients with large rebound effects had high pretreatment levels of BTMs, had received high cumulative doses of denosumab and had a good treatment response. On the contrary, patients with low pretreatment levels tended to lack rebound effects, and remarkably hardly any rebound was observed in a patient with high pretreatment levels but poor response to denosumab, although treated with the longest duration and highest cumulative dose. These observations may cautiously indicate that the degree of bone turnover before treatment combined with the magnitude of the skeletal improvement on treat-

ment are more important contributors to the rebound phenomenon than the duration or cumulative dose of denosumab. In patients with osteoporosis, the rebound after denosumab discontinuation appears to be larger in patients with a higher pretreatment bone turnover, after a longer treatment duration, or after higher-dose therapy, while pretreatment with bisphosphonates was found to be protective against a rebound, although consensus on this topic has not yet been reached[39, 42-48]. A hypothesis for the mechanism behind the rebound phenomenon is that an excessive remodeling rate occurs after withdrawal from denosumab. In mice it is observed that OPG, the natural decoy receptor for RANKL mimicked by denosumab, induced the fission of active, multinucleated osteoclasts into smaller, less active daughter cells termed 'osteomorphs' by McDonald et al. Upon withdrawal from OPG, the osteomorphs quickly reassembled into bone resorbing osteoclasts and bone resorption rebounded[49]. In humans after denosumab discontinuation a similar process may occur, leading to a decrease in trabecular bone volume more than in cortical bone volume, and to an elevated amount of unmineralized bone[44, 49-51]. Such a mechanism could be aggravated by a long treatment duration of Dmab, a high cumulative dose, or by a good response to therapy. Our results support this hypothesis, because patients with a good response and supposedly a high number of inactivated osteomorphs had a higher rebound and vice versa, although in our data the effect of treatment duration remains questionable. Another hypothesis is that of a mechanostatic reset, where a homeostatic, intrinsic mechanism defines the setpoint of bone remodeling in a patient, and where the suppressed bone turnover naturally regresses to this setpoint after discontinuation of antiresorptive therapy[52]. This theory is supported by two studies with osteoporosis patients, in which a correlation was observed between the gain in bone mineral density after Dmab therapy and the loss after discontinuation, although it has to be noted that patients with more severe disease generally require a longer treatment exposure in order to reach treatment targets[36, 48]. Specifically for FD/MAS this theory is of great interest, as both the activity and the extent of bone lesions are markedly variable between patients[35, 53]. Our data provides preliminary support for this theory, because in most patients the bone turnover markers returned to roughly around pretreatment levels, but further research is needed to confirm these findings and to provide further evidence for the mechanisms responsible for the relapse or rebound in bone turnover markers in FD/MAS. Another feature of the two patients in our study with the largest rebound effect was the presence of MAS. Likewise further research is needed to clarify whether the hormonal abnormalities in MAS are contributing to the rebound effect or were in our cohort merely a coexisting trait in these patients.

In addition, our results show that denosumab therapy had beneficial effects on bone turnover in the majority of patients in our cohort. In 2/3 of patients with elevated levels of ALP, P1NP or B-CTX normalization was observed during treatment. Moreover, all markers showed a significant and clinically relevant decrease after 1 year of treatment. The results also suggest a beneficial effect on bone pain, as maximum pain scores declined significantly by 2.7 points (95%CI: 0.8-3.6) in the first 2 years of treatment, a patient-determined clinically relevant response[54]. However, in future studies it should be investigated whether this finding resulted from a treatment effect or whether concepts such as regression to the mean or the placebo effect interfered with our results. Nevertheless important to reminisce is that pain scores alone are not sufficient to reflect the benefits of denosumab therapy. This is illustrated by 3 patients who felt more energetic despite unchanged pain levels. Moreover, 1 in 4 patients were able to reduce the dosage of or withdraw from analgesics.

Concerning adverse effects, in our cohort one patient (2.7%) developed osteonecrosis of the jaw after a dental procedure without antibiotics prophylaxis nor primary sutures of the wound. The risk for osteonecrosis of the jaw in FD/MAS on denosumab has never been studied but it is postulated that patients with a low skeletal burden score (SBS 10) have a higher risk for developing ONJ, because the large amount of non-FD bone is particularly at risk for ONJ[55]. Our results do not support this hypothesis, as the patient with ONJ had a SBS of 31.4. The risk for ONJ in our cohort is comparable to the incidence in patients receiving denosumab for solid tumors (1.7%)[56] and for osteoporosis (0-3.0%)[22, 57], although these risks are hard to compare due to variable patient characteristics, treatment schedules and research methods. Supposedly the risk is not higher than the risk for ONJ in FD/MAS patients treated with bisphosphonates where incidences from 0-5.4% have been reported[17, 58].

Our study is the largest on the treatment with denosumab in FD/MAS and the first to assess the safety of denosumab discontinuation. A limitation of our study is that BPIs and lab marker values were assessed at normal follow up before, during and after treatment and that timepoints may vary. Especially after withdrawal some patients have merely one or a few available measurements, which complicates the interpretation of the direction of the rebound and whether it is resolving or exacerbating. In addition in some patients the laboratory measurements were first evaluated several months after withdrawal, when it cannot be ruled out that an rebound occurred and resolved rapidly and unnoticed. Future studies with standardized laboratory measurements should address this issue, which are currently ongoing (clinicaltrials.gov: NCT03571191). Nevertheless, even when rebounding bone turnover markers would be present but missed,

we still did not observe clinically relevant consequences of high calcium levels, such as vomiting, or of an excessive bone remodeling, such as fractures. Another limitation is the lack of standardized treatment schemes in our patients. Patients were treated with either 60 or 120 of denosumab in intervals of 2, 3, 4 or 6 months according to their clinical and biochemical response. Currently, an initial treatment scheme of 60 mg denosumab every 3 months is recommended based our previous cohort study which analyzed a subset of the patients included in this present study [35]. On the other hand, this heterogeneity in treatment schemes as well as in patient characteristics makes this study generalizable to the clinical situation. Lastly, the amount of patients who ceased denosumab treatment was rather small, 14 patients, although this is still the largest case series until now. Small populations are a common problem in research on rare diseases. The absence of fractures in this cohort after discontinuation of denosumab is reassuring albeit a much larger cohort is needed to assess a risk similar to the risk for multiple vertebral fractures after withdrawal in case of osteoporosis [36, 40].

Notwithstanding these limitations, our results suggest a beneficial effect of denosumab treatment on pain, on the use of analgesics, as well as on bone turnover markers. Future research should determine whether denosumab is indeed more effective than placebo in relieving pain and whether denosumab could prevent the development or growth of FD lesions. Similarly, the incidence of osteonecrosis of the jaw needs further attention. In addition, the currently recommended treatment scheme should be investigated in a larger randomized controlled trial and the safety and efficacy of denosumab in children requires more research[55].

Concerning withdrawal, the results demonstrate that cessation of denosumab induces a biochemical rebound in bone turnover markers and possibly in calcium, but without significant morbidity, acute deterioration or hospital admittance. We cautiously suggest that withdrawal can be considered when denosumab treatment is no longer indicated. Nevertheless, denosumab should initially only be applied when bisphosphonates are not tolerated or not effective and only in a tertiary referral center with ample experience in the management of FD/MAS. After discontinuation, close biochemical and clinical surveillance is recommended and additional antiresorptive therapy should be considered. More research is needed into the extent of the observed rebound phenomenon, into risk factors as well as into the histologic response to cessation. Lastly, treatment regimens to prevent or treat discontinuation rebound should be explored.

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Chapter 9

Summary

Summary

Fibrous dysplasia/McCune-Albright syndrome (FD/MAS) is a chronic and possibly debilitating disorder, for which treatment may be challenging due to the heterogenic patient presentation, rarity of the disease, and variable treatment response. This thesis aimed to unravel several knowledge gaps in the pathophysiology, epidemiology, and progression of FD/MAS with basic, translational and cohort studies, and evaluated outcomes of treatment in order to enhance symptom management and patient wellbeing.

Part 1: Pathophysiology

In chapter 2, the first proof-of-concept study for human ex-vivo tissue cultures was discussed. Skeletal surgical waste tissues were explanted and cultured up to 7 days, and antigens of interest were detected with histological, enzymatic and immunohistochemical staining. Histomorphometric analyses demonstrated preserved tissue architecture, preserved functioning of early and late osteoblasts and of osteoclasts, with limited apoptosis. Sections showed representative characteristics of FD/MAS in comparison to control tissue. These results point to a successful culture model and allow future research with pharmaceutical agents.

Chapter 3 described a translational study, where concentrations of potential biomarkers Receptor Activator of Nuclear factor kB-ligand (RANKL), osteoprotegerin (OPG), sclerostin and interleukin-6 (IL-6) were measured in serum samples of 96 adult patients with FD/MAS. Correlations were calculated with standard bone turnover markers alkaline phosphatase (ALP), procollagen type 1 propeptide (P1NP) and beta crosslaps (CTX), skeletal burden score (SBS), and pain scores. RANKL serum levels correlated weakly with ALP (Spearman rho 0.309, p=0.004), but no other correlations were observed between the potential biomarkers and standard bone turnover markers and none of the studied markers correlated with pain, SBS, or bisphosphonate treatment response, showing no superiority over standard markers in assessing disease severity or activity and emphasizing the importance of nuclear imaging and patient-reported outcome measures. Yet, IL-6 and RANKL levels prior to denosumab treatment were higher in patients with adequate treatment response to denosumab compared to non-responders, and correlated with an improvement in pain scores. This observation in a small group of patients could aid in selecting patients who might benefit from denosumab treatment.

For chapter 4, the link between Receptor Activator of Nuclear factor kB-ligand (RANKL) upregulation and development of breast cancer in FD/MAS was ex-

plored. RANKL immunohistochemistry was performed in breast cancer tissue of 9 patients with FD/MAS, of matched control patients, and of pregnant positive control patients. Three patients with FD/MAS (38%) demonstrated RANKL expression versus 0 control patients. Staining was observed in certain areas of surrounding healthy ductal lobular, but barely in malignant tissue, with a higher positive area percentage in these 3 FD-patients compared to both control groups and with strong intensity comparably to positive controls. Those 3 patients with FD/MAS all had thoracic FD lesions but only 1/3 patients had a high FD skeletal burden. These results point to a local and possibly mosaic upregulation of RANKL in healthy mammary tissue in FD/MAS, which could induce an oncogenic niche in concurrence with other risk factors. Yet, the pathophysiology of RANKL-driven breast cancer needs to be elucidated, and questions remain on reasons for absence of expression in other FD patients, on the link between skeletal burden or other possible risk factors and RANKL expression, and on implications for early screening or prognosis.

Part 2: Disease characterization

In chapter 5, a cohort study was conducted with the nation-wide registry in Denmark. Patients were included with either ICD-10 code M85.0 for monostotic FD (n=269) or Q78.1 for polyostotic FD/MAS (n=139). Both incidence and prevalence of FD/MAS increased over the years with the most recent incidence rate in 2015-2018 being 3.6 per 1,000,000 person-years (95% CI: 2.9, 4.5) and prevalence 61.0 per 1,000,000 persons (95% CI: 54.6, 67.4) in 2018, which is equivalent to 1 case per 16,500 (95% CI: 14,837, 18,315) persons. FD/MAS was most frequently diagnosed between age 11 and 20 and incidence rate of MFD was 2.5-fold that of PFD/MAS at the end of the study period. This study is the first to calculate incidence and prevalence values of clinically apparent FD/MAS a nationwide cohort with low selection bias. These measures provide understanding of the distribution of the disease and are useful for patient counseling, health care planning and budgeting, comparison with other skeletal dysplasias, and for future studies.

For chapter 6, risk factors for progressive varus deformity in the proximal femur were determined in a cohort of pediatric and adolescent patients <30 years of age with femoral involvement of FD in two tertiary centers, the LUMC in the Netherlands and NIH in the USA. In 180 patients with 274 affected femurs, the prevalence of the coxa vara deformity was 36%. In nested case-control analysis comparing patients with and without deformity, risk factors were presence of MAS, hyperthyroidism, hypophosphatemia, high percentage of the femur affected by FD, and calcar destruction (all p-values <0.001). The linear mixed effects model demonstrated the following risk factors for progression of de-

formity: growth hormone excess (β = 7.2, p = 0.013), hyperthyroidism (β = 11.3, p < 0.001), >25% of the femur affected (β = 13.2, p = 0.046), calcar destruction (β = 8.3, p = 0.004), cystic appearance of the lesion (β = 3.9, p = 0.009), and bilateral involvement (β = 9.8, p = 0.010). Development of deformity accelerated in patients <15 years of age with neck-shaft angle declining <120 degrees. In this study, frequent monitoring is suggested of radiographic, lesional risk factors as well as systemic, patient-related risk factors for deformity. It is recommended to optimize treatment according to the guidelines and to consider surgery in teenagers and adolescents if the NSA declines below 120 degrees.

Part 3: Treatment

Chapter 7 focused on effects of 1 year of care according to a multidisciplinary care pathway for FD/MAS on pain, measured with the Brief Pain Inventory (BPI) by visual analogue scale (VAS) (0-10), and on quality of life, as measured with Short Form 36 (SF-36). The study demonstrated reduced scores for all domains of quality of life in patients with FD/MAS at baseline compared to the general population and moderate to severe pain in 67% of patients. Patients who were referred to the care trajectory < 1 year prior to completion of the baseline questionnaires reached a clinically important difference (CID) of 1 point on VAS for maximum pain, average pain and pain interference after 1 year or care, as well as a CID in domains Physical Function, Role Physical, Social Functioning, and Health Change. Patients who were already included in the care pathway > 1 year prior to baseline, also reported improved Physical Function and Emotional Well-being despite stable pain scores after 1 year. This study underwrites findings of previous studies that pain, functional disability and reduced quality of life are important aspects of FD/MAS yet difficult to manage, and suggests that referral to a tertiary care center with a dedicated pathway needs to be recommended.

In chapter 8, results of withdrawal from denosumab treatment were reported in 37 adult patients during a median follow-up of 3.2 years after withdrawal. Only one patient demonstrated hypercalcemia after discontinuation, yet mild, asymptomatic and self-limiting. An increase in ALP, P1NP and CTX was observed in the majority of patients mostly between 3 and 6 months after withdrawal, with levels exceeding pretreatment levels in 46% of cases for ALP, 44% for P1NP and 89% for CTX. The highest rebound was observed in patients with high pretreatment levels responding well to treatment, while patients with no suppression of bone turnover markers during treatment (non-responders) did not demonstrated change after discontinuation. Despite the biochemical rebound, no fractures, pain flares or lesions progression were observed, indicating that withdrawal from denosumab therapy may be safe, but warrants frequent clinical and biochemical follow-up.

Chapter 10

Discussion

Discussion

This thesis shed light on the pathogenesis of FD/MAS, characteristics of disease, and treatment outcomes. The following chapter discusses the results with its clinical implications and in the context of previously conducted research, and highlights unanswered topics with recommendations for future research.

Disease pathogenesis and drug targets

Many studies have investigated the complex pathophysiology of FD/MAS. For several years, the downstream cascade of the GNAS mutation in FD bone lesions has been known, consisting of subsequently overstimulation of cAMP and protein kinase A, increased c-fos and c-jun, and alterations in sclerostin en Wnt/β-cathenin pathway leading to fibrous hyperproliferative lesions, with important contributions of FGF-23 to bone mineralization and upregulation of RANKL, OPG and IL-6 to the coupling of bone production and bone resorption[1-3]. However, the specific importance of each of these proteins and many more is an ongoing quest, as well as the effect of suppression on these targets on lesion development or activity. Apart from the cellular pathways of mutated cells, new studies have explored paracrine, immunomodulatory and epigenetic effects[4-6]. Addressing these remaining questions is not only important for the understanding of the disease, but also for the development of new pharmaceutics, since the currently used bisphosphonates or denosumab therapy may alleviate symptoms, but are not effective in a subset of patients and do not cure the disease[7, 8]. The proportion of patients refractory to treatment together with the heterogeneity in patient presentation demands a personalized treatment strategy, yet this is hindered by the lack of knowledge on reasons for treatment non-responsiveness. Several studies with animal models, cellular models, or explanted tissues have tried to answer these questions, each with distinct advantages and disadvantages. The culture model as described in chapter 2 is the first ex-vivo culture model for FD/MAS and was set out to contribute to both the understanding of FD/MAS and the development of new, personalized therapy options. This proof-of-concept study successfully demonstrated adequate tissue viability and cell function for at least 7 days, which allows our research to proceed to the next step: administering new and currently used therapeutic agents and monitoring cellular response. In the future, this approach might offer patients personalized medicine based on the response of explanted tissue to administered drugs and which may eliminate the necessity to try several treatments with possible side effects but no symptom alleviation.

Markers of disease activity

The need for a histological model to investigate treatment response has partially been driven by the lack of serological markers suitable to assess disease activity or capture treatment response. Current markers for disease activity include alkaline phosphatase (ALP), osteocalcin, procollagen type 1 propeptide (P1NP) and beta crosslaps (CTX). These markers provide information on bone turnover as a proxy for disease activity, but do not correlate with pain, do not correlate with skeletal burden score (SBS) consistently across studies, and are not able to predict or monitor treatment response[8-10]. Previous studies have shown interest in other markers, yet with unsuccessful results[11]. Our study on serum RANKL, OPG, sclerostin and IL-6 described in chapter 3 showed heterogeneous levels but no benefit of these markers over the standard bone turnover markers in assessing disease burden or activity. However, elevated levels of IL-6, RANKL and elevated RANKL/OPG ratio might have some predictive value for clinical response to denosumab, but the sample size was small. For future studies it might be considered to evaluate serum levels of these markers prior, during and after treatment with denosumab to further explore this relationship. Nevertheless, our results underwrite the importance of assessing disease activity with nuclear imaging. Recent research have reported $Na^{18}F$-PET/CT as useful and objective tool for quantifying disease extent and lesional activity[12, 13]. In addition, Van de Bruggen et al. showed that $Na^{18}F$-PET/CT is able to capture response to denosumab treatment, since a decrease in lesional volume was seen after denosumab therapy, but no change after non-denosumab therapy or in healthy bone[14]. The observed decline in volumetric measurements correlated with bone turnover markers and pain reduction. Lesional volume even declined in patients with low skeletal burden at baseline and low bone turnover markers unaffected by therapy.

Tumor development as consequence of extraskeletal involvement of FD/MAS

Apart from the serological markers resulting from skeletal lesional activity, more questions remain on the systemic consequences of FD/MAS. Previous studies have shown an increased risk for benign and malignant tumors, including intramuscular myxomas, ovarian cysts, microlithiasis, testicular and cervical cancer, breast cancer, prostate cancer, thyroid cancer and melanoma, gastrointestinal, hepatobiliary and pancreatic neoplasms. GNAS mutations have been detected in some of these malignancies and were also demonstrated to induce ongoing cell growth in premalignant tissues, but an oncogenic role for RANKL has also been described both in relation to and outside the scope of FD/MAS[15-18]. Chapter 4 points to a link between RANKL upregulation and breast cancer, but it remains unknown why merely healthy and not malignant tissue exhibited RANKL

expression. In addition, our study could not assess whether RANKL upregulation was due to an endocrine effect of circulating RANKL resulting from skeletal disease, or a local upregulation, possibly caused by GNAS mutated ductal cells, creating an oncogenic niche. Future research will need to investigate the relation between GNAS mutations, RANKL upregulation, possibly other oncogenic factors, and tumor development. Currently, physicians need to be aware of the susceptibility to malignancies in patients with FD/MAS, to encourage patients to be compliant to national screening programs and avoid additional risk factors, and to facilitate additional imaging if required.

Management of bone deformities

Other difficult aspects in the treatment of FD/MAS include the prevention and management of deformities and pain. Craniofacial deformity may induce nerve compression and cosmetic issues, scoliosis may worsen over time and even lead to fatal restrictive lung disease if left untreated, and lower leg deformities may severely impair physical function. Overall, total skeletal burden, FGF-23 mediated hypophosphatemia, hyperthyroidism, growth hormone excess and elevated bone turnover markers have been associated with progression of deformity[19-21]. However, the effect of treatment of these factors on deformity is less clear. Our study in chapter 6 evaluated risk factors for deformity of the proximal femur, which is susceptible to progressive varisation and lateral bowing due to weight bearing forces. Coxa vara deformity may induce (micro)fractures, pain and physical disability, but is difficult to treat surgically due to implant failure related to the decreased mechanical bone strength, to implant difficulties in severely deformed bone, to the need for revision surgeries in progressive deformity, and to problems with implant size and the growing skeleton in pediatric patients[22, 23]. Our study also indicated severe disease, hyperthyroidism, hypophosphatemia and growth hormone excess as risk factors for progressive deformity, as well as local factors including lesion size, calcar destruction, and lesional radiolucency. This study provides a better understanding of the etiology of the coxa vara deformity, but did not evaluate whether treatment, yielding a reduction in lesion size or an improvement in cortical thickness or lesional mineralization, would prevent progression of deformity, relieve symptoms, or benefit surgical success. More research is needed on strategies to prevent lesion growth and to decelerate the development of deformity, and should address the optimal timing and technical approach for surgical correction of deformities in order to improve functional outcomes and quality of life.

Management of pain

Concerning the management of pain, multiple studies have investigated causal factors for pain. High lesional activity may provoke bone pain, although several papers report contradictory results on the relationship between pain scores and skeletal burden or bone turnover markers. Pain levels are higher in patients with lower extremity lesions compared to upper extremity disease, and higher in adults compared to children. Moreover, high levels of pain are associated with reduced physical function and impaired quality of life. It is important to recognize mechanical or neuropathic pain, since these types of pain are presumably less responsive to treatment with antiresorptive therapy. Mechanical pain could signal impending or stress fracture and may require surgical therapy, while neuropathic pain may be related to IL-6 upregulation, abnormal nerve sprouting or nerve fiber reorganization and may benefit from therapy with antidepressants or anti-epileptic medications such as amitriptyline, gabapentin or pregabalin[9, 24-26]. To address pain, quality of life, and all other patient-specific complaints in a multifactorial treatment strategy, sufficient expertise of different specialties is required. Therefore it has been suggested to concentrate care for patients with FD/MAS in tertiary care facilities. Chapter 7 of the thesis demonstrated that 1 year of treatment in a multidisciplinary coordinated care pathway improved scores for several domains of quality of life and for pain. Because of these results, combined with the rarity and heterogeneity of the disease and the need for further research, we propose management of symptomatic FD/MAS only in specialized centers.

Moreover, treatment with denosumab is only safe when applied by clinicians with sufficient expertise. Denosumab has demonstrated beneficial effects on pain, bone turnover markers, lesion development and progression and lesion mineralization. Yet, concerns have been raised regarding rebounds effects after withdrawal, following two case reports of children with severe and symptomatic hypercalcemia after denosumab cessation[7, 8, 14]. Our study in chapter 8 has confirmed that denosumab therapy may safely be discontinued in adults, with only mild and asymptomatic rebound effects in bone turnover markers and calcium. Presumably the degree of the rebound phenomenon is associated with high skeletal burden, high treatment response and young age of the patient. Yet, if denosumab could be administered in young children and halt disease progression, it could possibly reduce the disease burden throughout life. Future research should look into indications for use in children and into measures to prevent and detect severe rebound effects.

Future perspectives

Overall, future studies should focus on clarifying the pathophysiology of skeletal and extraskeletal consequences of FD/MAS and on mechanisms contributing to pain, by optimizing the use of patient-derived tissues and modelling the influence of endocrinopathies and of therapeutic agents on lesional activity and bone strength. This might consequently reveal new serum markers as proxy for disease activity. However, since previous research into serum biomarkers has been unsuccessful but into $Na^{18}F$-PET/CT promising, indications for and clinical usefulness of nuclear imaging should be investigated in large cohort studies. Moreover, disease characteristics need to be investigated in both pediatric and adult patients, including early signs of disease progression and factors influencing pain, physical function and quality of life. Improved understanding of the etiology and characteristics of FD/MAS combined with new therapeutic options would benefit early and personalized treatment. The heterogeneity, complexity and rarity of the disease demands a multidisciplinary approach, including psychosocial support, in tertiary referral centers, especially for patients with severe complaints, polyostotic disease or MAS, to achieve optimal outcomes. International collaborations are necessary to share expertise in clinical management and obtain larger patient cohorts for research.

General conclusions

Part 1: Pathophysiology

- This thesis aimed to explore histological features, tissue viability and cell functioning in an ex-vivo culturing model. FD bone lesions demonstrate irregular, thin, undermineralized trabecular bone with hypercellular fibrous marrow with abundant early osteoblasts. Cultured tissues show preserved cell viability and function up to 7 days.
- Clinical usefulness of potential serum biomarkers RANKL, OPG, IL-6 and sclerostin was assessed. Our data show no correlation of these markers with FD/MAS disease activity, pain, skeletal burden score, or response to bisphosphonate therapy, and therefore no superiority of these new markers to currently used markers of bone turnover.
- RANKL expression was measured in breast cancer specimens and compared between patients with and without FD/MAS. This thesis demonstrated RANKL upregulation in healthy mammary tissue surrounding cancerous lesions in certain patients with FD/MAS.

Part 2: Disease characterization

- A nation-wide registry in Denmark was used to assess the epidemiology of FD/MAS. The incidence rate of FD/MAS is 3.6 per 1,000,000 person-years (95% CI: 2.9, 4.5), while the prevalence is 61.0 (95% CI: 54.6, 67.4) per 1,000,000 persons, corresponding to 1 case per 16,500 (95% CI: 14,837, 18,315) persons.
- This thesis set out to identify risk factors for progressive varus deformity of the proximal femur in FD. Varus deformity occurs in 36% of cases and risk factors for deterioration in deformity include hyperthyroidism, hypophosphatemia and growth hormone excess, large lesions, calcar destruction, and radiolucency.

Part 3: Treatment

- The value of treatment of FD/MAS in a multidisciplinary care pathway in a tertiary center was evaluated. 1 year of treatment in this care trajectory benefits quality of life in both newly referred patients and patients under chronic care, and reduces pain in newly referred patients.
- Safety of withdrawal of denosumab therapy was studied in adult patients. Therapy discontinuation may induce an asymptomatic and self-limiting biochemical rebound phenomenon, but is generally safe, without occurrence of fractures, pain flares or lesion progression.

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Appendices

Dutch summary

Portfolio

Curriculum vitae

Acknowledgements

Nederlandse samenvatting

Fibreuze dysplasie/McCune-Albright syndroom is een chronische en potentieel invaliderende aandoening die lastig te behandelen kan zijn, met name vanwege de heterogene patiëntenpopulatie, de variabele behandeluitkomsten, en de zeldzaamheid van de ziekte. Het doel van dit proefschrift was om verscheidene kennishiaten in de pathofysiologie, de epidemiologie en het ziekteverloop van FD/MAS te onderzoeken door middel van basale, translationele en cohort studies. Tevens zijn in dit proefschrift uitkomsten van behandelingen geanalyseerd, met als uiteindelijke doel het verminderen van symptomen en het verbeteren van kwaliteit van leven van patiënten.

Deel 1: Pathofysiologie

Hoofdstuk 2 beschreef de eerste haalbaarheidsstudie naar het kweken van geëxplanteerd humaan weefsel. Restweefsel van skeletlaesies werd verzameld en gekweekt tot 7 dagen, waarna relevante antigenen werden aangetoond door middel van histologische, enzymatische en immunohistochemische kleuringen. Histomorfometrische analyses lieten zien dat de weefselarchitectuur intact bleef en de functie van vroege en late osteoblasten en van osteoclasten behouden, met beperkte apoptose. De weefselcoupes van FD-patienten toonden typische karakteristieken van fibreuze dysplasie in vergelijking met gezond weefsel. Deze resultaten laten zien dat het kweken succesvol is geweest en dat dit model de volgende stap in het onderzoek mogelijk maakt, namelijk het evalueren van het effect van medicatie op het botweefsel.

Hoofdstuk 3 belicht een translationele studie, waarin concentraties van potentiele biomarkers Receptor Activator of Nuclear factor kB-ligand (RANKL), osteoprotegerin (OPG), sclerostin en interleukin-6 (IL-6) werden gemeten in het serum van 96 volwassen patienten met FD/MAS. Correlaties werden berekend met de standaard botmarkers alkalisch fosfatase (ALP), procollageen type 1 propeptide (P1NP) en beta crosslaps (CTX), met ziektelast uitgedrukt in skeletal burden score (SBS), en met pijnscores. Een zwakke correlatie werd geobserveerd tussen RANKL en ALP (Spearman rho 0.309, p=0.004), maar verder correleerden de potentiële biomarkers niet met de standaard botmarkers, niet met pijn of SBS en ook niet met de respons op behandeling met bisfosfonaten. Deze biomarkers kunnen de ziektelast en ziekteactiviteit dus niet beter aantonen dan de standaard botmarkers. Dit benadrukt het belang van nucleaire beeldvorming en patiënt-gerapporteerde uitkomsten. Echter, concentraties van IL-6 en RANKL voorafgaand aan denosumab behandeling waren hoger in patienten met een goede therapie respons in vergelijking met de non-responders, en correleerden met de verbetering in pijnscores na behandeling. Deze bevinding in een kleine

groep patienten zou kunnen bijdragen aan het selecteren van patiënten die mogelijk baat hebben bij behandeling met denosumab.

In hoofdstuk 4 werd de link onderzocht tussen RANKL overexpressie en borstkanker in patienten met FD/MAS. RANKL immuunhistochemie werd uitgevoerd op borstkankerweefsel van 9 patienten met FD/MAS, van gematchte controlepatiënten, en van zwangere patienten als positieve controle. RANKL expressie werd gezien in drie patienten met FD/MAS (38%) versus 0 controlepatiënten. De aankleuring werd geobserveerd in specifieke gebieden van omliggend gezond ductaal en lobulair weefsel maar nauwelijks in maligne weefsel. Het percentage positieve gebieden was in deze 3 FD-patienten hoger dan in beide controlegroepen, en de intensiteit van de kleuring even sterk als in de positieve controles. Deze 3 patienten met FD/MAS hadden allen thoracale skeletlaesies maar slechts 1 van de 3 patienten had een hoge ziektelast. Deze resultaten duiden op een lokale dysregulatie van RANKL, mogelijk in een mozaïekpatroon, in gezond borstweefsel in FD/MAS. Dit zou in combinatie met andere risicofactoren kunnen leiden tot een oncogene omgeving. Desalniettemin moet de pathofysiologie van RANKL-gedreven borstkanker verder uitgediept worden. Het blijft onduidelijk waarom geen RANKL expressie werd gedetecteerd in de overige patienten met FD, wat de relatie is tussen ziektelast of andere risicofactoren en RANKL expressie, en welke implicaties dit geeft voor vroege screening of voor de prognose.

Deel 2: Ziekte karakteristieken

In hoofdstuk 5 werd een cohort studie uitgevoerd met het nationale register in Denemarken. Patienten werden geïncludeerd met ICD-10 diagnosecode M85.0 voor monostotische FD (n=269) of Q78.1 voor polyostotische FD of MAS (n=139). Een stijging in zowel incidentiecijfer als prevalentie werd gezien over de tijd. De meest recente incidentie in 2015-2018 was 3.6 per 1,000,000 persoonsjaren (95% CI: 2.9, 4.5) en prevalentie 61.0 per 1,000,000 personen (95% CI: 54.6, 67.4) in 2018, equivalent aan 1 patiënt per 16,500 (95% CI: 14,837, 18,315) personen. De diagnose FD/MAS werd het vaakst gesteld in de leeftijdscategorie 11 tot 20 jaar. Het incidentiecijfer van MFD was 2.5 keer zo hoog als van PFD/MAS aan het einde van de onderzoeksperiode. Dit is de eerste studie die incidentie en prevalentie van klinisch gediagnosticeerde FD/MAS heeft gekwantificeerd in een nationaal cohort met beperkte selectiebias. Deze maten geven informatie over de distributie van de ziekte en zijn nuttig voor uitleg aan patienten, voor het plannen en budgetteren van gezondheidszorg, voor het vergelijken van FD/MAS met andere skeletdysplasieën, en voor toekomstig onderzoek.

In hoofdstuk 6 werden risicofactoren bepaald voor progressieve varus deformiteit van het proximale femur in een cohort met pediatrische en adolescente patienten van 30 jaar of jonger met ziektelokalisaties in het proximale femur. De studie werd uitgevoerd in 2 tertiaire centers: het LUMC in Leiden en de NIH in de Verenigde Staten. De prevalentie van de coxa vara deformiteit was 36% in 180 patienten met 274 aangedane femurs. Een geneste case-controle-analyse, waarin patienten met en zonder deformiteit werden vergeleken, liet de volgende risicofactoren zien: aanwezigheid van MAS, hyperthyreoïdie, hypofosfatemie, hoog percentage van het femur aangedaan door FD, en calcar destructie (alle p-waarden <0.001). Risicofactoren voor progressieve deformiteit in een lineair mixed-effects model waren: groeihormoon overproductie ($\beta = 7.2$, $p = 0.013$), hyperthyreoïdie ($\beta = 11.3$, $p < 0.001$), >25% van het femur aangedaan ($\beta = 13.2$, $p = 0.046$), calcar destructie ($\beta = 8.3$, $p = 0.004$), cysteus aspect van de laesie ($\beta = 3.9$, $p = 0.009$), en bilaterale betrokkenheid ($\beta = 9.8$, $p = 0.010$). De deformiteit was met name progressief in patienten jonger dan 15 jaar met een afnemende caput-collum-diafysehoek onder 120 graden. Deze studie adviseert frequente monitoring van radiografische, laesie-gerelateerde risicofactoren alsmede van systemische, patiënt-gerelateerde risicofactoren voor deformiteit. Het wordt aangeraden om behandeling te optimaliseren conform de richtlijnen en om chirurgische behandeling in jongeren en adolescenten te overwegen indien de caput-collum-diafysehoek onder 120 graden daalt.

Deel 3: Behandeling

Hoofdstuk 7 richtte zich op het effect van 1 jaar zorg volgens een multidisciplinair zorgpad voor FD/MAS op pijn, gemeten met de Brief Pain Inventory (BPI) middels visueel analoge schaal (VAS) (0-10), en op kwaliteit van leven, gemeten met de Short Form 36 (SF-36). In deze studie scoorden patienten met FD/MAS op baseline lager dan de algemene populatie op alle domeinen van kwaliteit van leven en rapporteerde 67% van de patienten matig tot ernstige pijn. Patienten die verwezen werden naar het zorgpad binnen 1 jaar na invullen van de baselinevragenlijst bereikten na 1 jaar zorg een klinisch relevante afname van 1 punt op de VAS voor maximale pijn, gemiddelde pijn, en belemmering door pijn en rapporteerden klinisch relevante verbeteringen in domeinen Fysiek functioneren, Rolbeperkingen door fysieke problemen, Sociaal functioneren en Gezondheidsveranderingen. Patiënten die bij het invullen van de baselinevragenlijst al langer dan 1 jaar behandeld werden volgens het zorgpad, rapporteerden na 1 jaar ook verbeteringen in Fysiek functioneren en Geestelijke gezondheid, ondanks dat pijnscores hetzelfde bleven. Dit onderzoek onderstreept de bevindingen uit eerdere studies dat pijn, functionele beperkingen en een verminderde kwaliteit van leven belangrijke, maar moeilijk behandelbare aspecten zijn van FD/MAS,

en suggereert dat verwijzing naar een tertiair centrum met een gespecialiseerd behandeltraject aangeraden dient te worden.

In hoofdstuk 8 werden resultaten gerapporteerd van het beëindigen van de behandeling met denosumab in 37 volwassen patiënten tijdens een mediane follow-up van 3.2 jaar na de laatste gift. Slechts 1 patiënt vertoonde hypercalciëmie na het voltooien van de therapie, echter mild, asymptomatisch en zelflimiterend. Een stijging in de labwaarden ALP, P1NP en CTX werd geobserveerd in de meerderheid van de patienten, meestal tussen 3 en 6 maanden na stoppen, met waarden hoger dan voor de behandeling in 46% van de patienten voor ALP, 44% voor P1NP and 89% voor CTX. De sterkste rebound werd gezien in patienten met hoge botmarkers vooraf en een sterke daling na starten van denosumab, terwijl patienten met stabiele waarden tijdens de behandeling (non-responders) ook geen rebound toonden. Ondanks de biochemische rebound werden geen fracturen, opvlamming van pijn, of ziekteprogressie gezien. Deze resultaten lieten zien dat denosumab veilig gestopt kan worden onder frequente klinische en biochemische controles.

Portfolio

Publications of this thesis

Meier ME, Van der Mark MH, Van der Horst G, Van de Sande MAJ, Bulaicon OO, Van der Pluijm G, Bravenboer N, Appelman-Dijkstra NM. Human ex-vivo tissue culture model for Fibrous Dysplasia/McCune-Albright syndrome – a proof-of-concept study. Manuscript in preparation.

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Meier ME, van der Bruggen W, van de Sande MAJ, Appelman-Dijkstra NM. Regression of fibrous dysplasia in response to denosumab therapy: A report of two cases. Dutch Society for Calcium and Bone Metabolism (NVCB), annual congress, 2020, online **(oral presentation)**

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Meier ME, Appelman-Dijkstra NM. Denosumab in Bisphosphonate resistant FD/MAS. Dutch ASBMR days, 2021, online **(oral presentation)**

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Grants

Research grant of €480.000 for conducting the study: "DEnosumab for the treatment of FIbrous Dysplasia/McCune-AIbright Syndrome in adults (DeFiD): a randomized double-blind placebo-controlled trial". Principle investigator: Natasha Appelman-Dijkstra. Provided by Dioraphte.

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Personal travel Grant of €500 for attending the CBM congress in 2019 in Athens, Greece. Provided by the Hellenic Society for the Study of Bone Metabolism & Dutch Society for Calcium & Bone Metabolism.

Courses

Basic methods and reasoning in Biostatistics – 2019

Basic course on Regulations and Organisation for clinical investigators (BROK) – 2019 (updated 2023)

Regression analyses – 2020

Clinical epidemiology – 2020

Academic Writing for PhDs – 2021

Speed reading – 2021

Presenting skills – 2021

Analysis of repeated measurements – 2022

Curriculum vitae

Maartje Ekwia Meier was born on June 24 in 1992 in Heerlen, as daughter of Marleen Meier-Adriaensens and Aswin Meier. Together with younger sister Eva and brother Bram, they lived in Valkenburg en later in Maasbree. Maartje completed Gymnasium at the Bouwens van der Booije college cum laude in 2010. In the same year she started studying Medicine at the University of Leiden. During college she contributed to studies on migraine as a research assistant at the Department of Neurology. She travelled to Nepal and Tibet for an internship Pediatric Surgery at the Kanti Children's Hospital in Kathmandu, and to Peru and Bolivia prior to commencing clinical internships. After graduating, she worked as non-training resident at the Department of Orthopedic Surgery at Alrijne Hospital and Amstelland Hospital. The PhD on fibrous dysplasia/McCune-Albright syndrome started in 2019, a combined trajectory of the Departments of Orthopedic Surgery and Internal Medicine, Division of Endocrinology of the Leiden University Medical Center, under supervision of prof. dr. Natasha Appelman-Dijkstra, prof. dr. Sander Dijkstra and later prof. dr. Michiel van der Sande. During the COVID-19 pandemic she worked shifts at the COVID-ward in the LUMC. Furthermore, she educated medical students in classes and working groups and supervised multiple research internships. During the PhD trajectory she obtained several grants, i.e. a personal grant of the European Programme for Rare Diseases for a research mobility fellowship, for which she moved to Aarhus in Denmark for a few months to work with the Department of Clinical Epidemiology and Department of Endocrinology & Internal Medicine of Aarhus University. She also contributed to the initiation of a multicenter randomized controlled trial on denosumab for FD/MAS, by preparing the study protocol and obtaining a research grant of €480.000 of Dioraphte together with a team of clinicians and with Natasha Appelman-Dijkstra as principle investigator. Maartje started the residency Orthopedic Surgery at the ROGO Rotterdam in 2023. After working as resident in general surgery in Reinier de Graaf Hospital and in orthopedic surgery in Erasmus Medical Center, she is currently working at Elisabeth Tweesteden Hospital in Tilburg.

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