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Original Research

High frequency of heterozygous rare variants of the *SLC34A1* and *SLC9A3R1* genes in patients with atypical femur fracture

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Abstract

Objective: Atypical femur fractures (AFFs) are rare fragility fractures originating at the lateral cortex of the femur, affecting the subtrochanteric or diaphyseal area of thebone with a transverse morphology. Occurrence of AFF is specifically associated with a small number of rare monogenic congenital metabolic bone disorders, such as hypophosphatasia, and with long-term treatment with antiresorptiondrugs. The exact pathogenesis of these fractures remains poorly understood and, except for cases of diagnosed HPP or other AFF-causing bone diseases, it is not possible to assess which patients are at higher riskof developing AFFs as a consequence of anti-resorption therapy.

Design: We genetically screened 25 unrelated patients who had developed at least one AFF.

Intervention: Genetic screening was performed through a nextgeneration sequencing analysis with a customized panel containing 76 human genes involved in the regulation of the mineralization processWe genetically screened 25 unrelated patients who had developed at least one AFF.

Results: We found a relatively high frequency (32.0%) of heterozygous rare variants inthe SLC34A1 and SLC9A3R1 genes, two genes whose heterozygous inactivating mutations have been respectively associated with autosomal dominant hypophosphatemic nephrolithiasis/osteoporosis types 1 and 2 (NPHLOP1and NPHLOP2). Other heterozygous rare variants were found in the BMPR1B, CYP27B1, FBN1, MEPE, PIGO, and PHOSPHO1 genes, each in a single AFF case (4.0%).

Conclusions and relevance: Our findings suggest that rarevariants of SLC34A1 and SLC9A3R1 could represent a possible genetic risk factor for the occurrence of AFFs. On the other hand, AFFs could represent an unsuspected clinical manifestation and/or an anti-resorption therapycorrelatedadverse event in patients with NPHLOP disorders.

Keywords: atypical femur fractures (AFFs), bone matrix mineralization, *SLC34A1* gene, *SLC9A3R1* gene, autosomal dominant hypophosphatemic nephrolithiasis/osteoporosis (NPHLOP)

Significance

Genetic testing is useful to identify unexpected genetic variants that could be responsible for specific clinical phenotypes, helping determine the correct clinical and therapeutic management of patients. Except for cases of diagnosed hypophosphatasia or a small number of atypical femur fracture (AFF)-causing Mendelian bone rare diseases, no other AFF-predisposing genetic causes have been identified to date, preventing pharmacogenetic possibilities to predict patients who should avoid anti-resorption therapies. Next generation sequencing analysis of AFF patients, with a multigenic panel specifically designed for mineralization, such as the one used in this study, identified rare genetic variants in unsuspected genes and, interestingly, found a relatively high frequency of heterozygous rare variants in the *SLC34A1* and *SLC9A3R1* genes, revealing possible novel genetic factors underlying the individual risk of AFF occurrence.

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Introduction

Atypical femur fractures (AFFs) are minimal- or no trauma-derived fractures, originating at the lateral cortex of the femur and affecting the subtrochanteric or diaphyseal area of the bone with a transverse or short oblique morphology, thus being easily distinguishable from osteoporotic fractures that occur at the intertrochanteric femur or the femoral neck. Prodromal pain and delayed fracture healing commonly manifest in patients with AFFs.[1]

The exact pathogenesis of these rare fractures is still poorly understood. Their stress-derived nature is consistent with a bone tissue subjected to repetitive loading that overwhelms the normal capacity of bone repair. Under normal circumstances, stress fractures are healed by localized bone remodeling. However, the presence of an unfavorable femur mechanical environment, and/or a genetically or pharmacologically determined impaired bone remodeling can increase the risk of AFF. In the first case, individuals with increased femoral neck varus or anterolateral bowing are prone to experience significantly increased tensile stresses at the femur lateral cortex during the normal body weight bearing, thus having a higher risk of AFF occurrence.[2,3] In the latter case, individuals with a genetically and/or pharmacologically determined impaired bone turn-over may have a delayed/reduced healing process of stress-determined microfractures that leads to fracture propagation, mostly in bone high-stress regions like the lower limbs, increasing the risk of AFF development. Occurrence of AFF has been specifically associated with some rare monogenic congenital metabolic bone disorders characterized by impaired bone mineralization and/or altered bone metabolism,[4,5] and with long-term treatments with two of the most commonly used anti-resorption drugs (bisphosphonates and denosumab), which specifically block osteoclast-driven bone resorption and reduce bone remodeling rate and healing.[6–8] Interestingly, biopsies of proximal femoral cortical bone adjacent to the fracture site from women with AFF treated with bisphosphonates have been shown to be harder and more mineralized than those from bisphosphonate-treated women with typical osteoporotic fractures, and long-term bisphosphonate treatment degraded the fracture-resistance toughening mechanisms typical of a healthy bone, suggesting the possibility of a synergistic effect of the use of such osteoclast-blocking drugs in determining an even higher risk of AFF in patients with a genetically predetermined impaired bone remodeling.[9]

In addition to the identification of variants in genes causing rare monogenic bone diseases in which AFFs have occurred, some studies have identified low-frequency variants in various genes involved in regulation of bone metabolism.[4,5,10]

Identifying genetic variants associated with the development of AFF may be of importance in the pharmacogenetic management of bone therapies, aimed to avoid the administration of anti-resorption drugs to those patients in which such molecules can further increase a genetic-derived risk of AFF.

Therefore, since genetically-driven altered mineralization process appears to be one of the main causes of AFFs, we decided to primarily focus our investigation on genes known to be involved in the regulation of bone mineralization, by genetically testing 25 unrelated AFF cases, which were previously resulted negative for the presence of a pathogenic mutation in the *ALPL* gene,[11] through a next generation sequencing (NGS)-based multigenic analysis of 76 mineralization-regulating genes.

Materials and methods

Patients

The study population included 25 patients (23 women and 2 men), collected at 3 clinical centers (Florence, Naples, and Geneva), who had manifested at least 1 AFF (mean age of fracture occurrence: 77.0 ± 19.7 years), and not presenting any geometrical and/or structural biomechanical alterations of the femurs as possible cause of AFF occurrence. Classification of AFF in our patients was performed at each clinical center by a radiological assessment, according to 4 major criteria, which were originally described by the Task Force of the American Society for Bone and Mineral Research (ASBMR) in 2010 and updated in 2014: (1) location of the fracture in the subtrochanteric region or diaphysis of the femur; (2) lack of trauma history; (3) a transverse or short oblique configuration; (4) nor- or minimal comminuted.[12,13] Fractures in the subtrochanteric or diaphyseal regions of the femur caused by falling and/or high-impact trauma have been excluded.

Twenty-four patients had previously resulted negative for *ALPL* inactivating mutations by Sanger's sequencing of coding region and splicing sites of the gene, while in 1 patient (AFF22) a non-polymorphic heterozygous missense variant of unknown clinical significance was identified (VUS) (p.Thr273Met).[11]

Main clinical characteristics of the 25 AFF patients are reported in Table 1.

A control population of 150 elderly subjects (65–90 years) was tested to identify rare variants. These control cases were randomly selected from patients attending the Outpatient Clinic of the Metabolic Bone Disease Unit of the Azienda Ospedaliero-Universitaria Careggi of Florence, from 2014 to 2017, for the evaluation of bone and mineral metabolism, and who gave their consent to collect a blood sample for future genetic analyses. The selected population included individuals with osteoporosis, osteopenia or normal bone mineral density, of whom 97 (64.7%) were under anti-fracture pharmacological treatment at the time of blood collection, including bisphosphonates. Patients with a history of any femur fracture (including both atraumatic and traumatic femur fractures) were excluded from the control group of this study.

Next generation sequencing analysis

NGS analysis was performed using a customized multigenic "mineralization panel", designed, developed, and tested through scientific and technical collaboration between the Italian Foundation for Research on Bone Diseases (F.I.R.M.O.) and Personal Genomics. The panel consisted of 76 human genes, including genes reported in the Online Mendelian Inheritance in Man (OMIM) as associated with congenital diseases affecting bone mineralization, and genes selected, through a target search of scientific literature, to be involved in the regulation of the mineralization process (Table S1).

Genomic DNA was extracted from venous blood with Qiaamp mini kit (Qiagen GmbH, Hilden, Germany). DNA libraries were produced using Kapa HyperPlus kit; target enrichment was performed using Kapa HyperChoice kit according to Kapa HyperCap protocol (Roche, Basilea, Switzerland). DNA libraries were quality checked using Labchip DNA High Sensitivity assay (Perkin Elmer,

Table 1. Main clinical characteristics of the 25 AFF patients.

Patient code	Identified rare genetic variant(s) (Gene)	Gender	Age at AFF manifestation	Characteristic of AFF(s)	History of fracture, other than AFF	History of nephrolithiasis and/or nephrocalcinosis	Osteoporosis or osteopenia (Age at DXA screening)	Serum activity of total ALP	Serum BALP value	Serum total calcium value	Urinary calcium value	Serum phosphate value	Urinary phosphate value	Anti-resorption therapy with bisphosphonates or denosumab before and/or at the time of AFF occurrence
AFF1	No rare genetic variant identified	F	n.a.	n.a.	n.a.	n.a.	n.a.	Slightly high	n.a.	Normal	n.a.	n.a.	n.a.	Yes (5-year discontinuous therapy with alendronate)
AFF2	c.328C > G; p.Leu110Val (*SLC9A3R1*	F	n.a.	n.a.	n.a.	n.a.	n.a.	Normal	n.a.	Normal	n.a.	n.a.	n.a.	n.a.
AFF3	No rare genetic variant identified	F	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
AFF4	No rare genetic variant identified	F	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
AFF5	No rare genetic variant identified	F	59	Fracture in the diaphyseal region of the left femur. Prodromic pain was reported before the AFF occurrence.	No	No	Osteoporosis at the lumbar spine (59 years) Osteopenia at the unfractured femur neck and total femur (60 years)	Normal	Normal	Normal	Normal	Normal	Normal	Yes (5-year discontinuous therapy with alendronate)
AFF6	No rare genetic variant identified	F	73	n.a.	Yes (Atraumatic vertebral fractures of D5, D6, D7, D8, D9, D10 and D12)	n.a.	Osteopenia at the lumbar spine (80 years)	n.a.	Normal	Normal	Normal	Low	Low	Yes (Denosumab)
AFF7	No rare genetic variant identified	F	65	Fracture in the diaphyseal region of the right femur, associated with prodromic pain.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
AFF8	c.782G > A; p.Arg261His (*SLC34A1*)	F	53 and 59	Atraumatic fracture at the subtrochanteric region of the right femur, on the same site of previous traumatic fracture, at 53 years. Atraumatic perthrocanteric fracture in the diaphyseal region of the right femur, occurring just walking at 53 years. Subthrocanteric fracture of the right femur, with a very low-energy	Yes (Traumatic fracture at the subtrochanteric region of the right femur at the age of 52, after falling during skying)	No	Osteoporosis at the lumbar spine and left femur neck (53 years)	Normal	Normal	Normal	Normal	Slightly high	Normal	Yes (Alendronate therapy up to the year before the occurrence of the first AFF)

(*continued*)

Table 1. Continued

Patient code	Identified rare genetic variant(s) (Gene)	Gender	Age at AFF manifestation	Characteristic of AFF(s)	History of fracture, other than AFF	History of nephrolithiasis and/or nephrocalcinosis	Osteoporosis or osteopenia (Age at DXA screening)	Serum activity of total ALP	Serum BALP value	Serum total calcium value	Urinary calcium value	Serum phosphate value	Urinary phosphate value	Anti-resorption therapy with bisphosphonates or denosumab before and/or at the time of AFF occurrence
				trauma, at 59 years. All associated with slow fracture healing and prodromic pain of the right leg.										
AFF9	c.616T > C; p.Cys206Arg (*SLC9A3R1*) c.1366C > T; p.Arg456Trp (*BMPR1B*)	M	36	Stress fracture of the diaphyseal region of the left femur; prodromic pain was reported before fracture occurrence.	Yes (Stress fracture of the left pubic bone, adjacent to the femur epiphysis at 37 years; prodromic pain was reported before fracture occurrence. Spontaneous microfracture of metatarsal bone at 38 years)	No	Osteopenia at the lumbar spine, right femur neck and total right femur (36 years)	Normal	Normal	Normal	Normal	Normal	Normal	No
AFF10	c.626A > G; p.Asn209Ser (*PIGO*)	F	71	Oblique fracture of the diaphyseal region of the left femur.	No	No	Osteopenia at the lumbar spine, femur neck and total femur (62 years)	n.a.	Normal	Normal	Dicontinuously low	Normal	Normal	Yes (10-year therapy with alendronate stopped 5 years before AFF occurrence. Therapy with denosumab started the year before AFF occurrence)
AFF11	No rare genetic variant identified	F	73	Atraumatic fracture of the diaphyseal region of the right femur.	No	n.a.	Osteoporosis at the lumbar spine, left femur neck and total femur (74 years)	Normal	Normal	Normal	Normal	Normal	n.a.	Yes (Long-term therapy with bisphosphonates before the occurrence of AFF)
AFF12	c.458G > A; p.Arg153Gln (*SLC9A3R1*)	F	4	Atraumatic fracture of the diaphyseal region of the right femur.	No	No	Osteoporosis at the lumbar spine and osteopenia at the femur neck and total femur (7 years)	Normal	Normal	Normal	Normal	Normal	Normal	No
AFF13	No rare genetic variant identified	F	77	n.a.	Yes (Atraumatic vertebral fracture of L3 at 72 years)	n.a.	Osteopenia at the lumbar spine and normal BMD value at femur neck and total femur (77 years)	Low	Dicontinuously low	Normal	n.a.	Normal	n.a.	Yes (4-year therapy with zoledronic acid)

(*continued*)

Table 1. Continued

Patient code	Identified rare genetic variant(s) (Gene)	Gender	Age at AFF manifestation	Characteristic of AFF(s)	History of fracture, other than AFF	History of nephrolithiasis and/or nephrocalcinosis	Osteoporosis or osteopenia (Age at DXA screening)	Serum activity of total ALP	Serum BALP value	Serum total calcium value	Urinary calcium value	Serum phosphate value	Urinary phosphate value	Anti-resorption therapy with bisphosphonates or denosumab before and/or at the time of AFF occurrence
AFF14	c.670G > T; p.Ala244Ser (*CYP27B1*)	F	58	n.a.	Yes (Metacarpal fracture. Wrist fracture at the age of 10. Atraumatic vertebral fractures of D11, D12, L1, and L4)	No	Normal BMD value at the lumbar spine. Osteoporosis at the femur neck and osteopenia at the total femur (58 years)	Normal	n.a.	Normal	n.a.	Normal	n.a.	Yes (6-year therapy with alendronate)
AFF15	No rare genetic variant identified	F	78	n.a.	Yes (Fragility fracture of the wrist)	n.a.	Osteopenia at the lumbar spine, femur neck and total femur (78 years)	Low	n.a.	Normal	n.a.	Low	n.a.	Yes (20-year therapy with alendronate)
AFF16	No rare genetic variant identified	F	88	n.a.	Yes (Wrist, proximal homerus and pelvic bone)	n.a.	Osteopenia at the lumbar spine and severe osteoporosis at the femur neck (T-score -3.5) and total femur T-score -3.3) (89 years)	Normal	n.a.	Normal	n.a.	Low	n.a.	Yes (10-year therapy with alendronate followed by 4-year therapy with denosumab)
AFF17	No rare genetic variant identified	F	62	n.a.	Yes (Ankle at the age of 58 years)	n.a.	n.a.	Normal	Normal	Normal	n.a.	Normal	n.a.	Yes (7-year therapy with alendronate followed by 2-year therapy with pamidronate)
AFF18	No rare genetic variant identified	F	81	n.a.	Yes (Traumatic vertebral fracture. Fracture of the wrist at 81 years)	n.a.	n.a.	Normal	n.a.	Normal	n.a.	Low	n.a.	Yes (4-year therapy with ibandronate)
AFF19	c.142C > G; p.Arg48Gly (*PHOSPHO1*)	M	51	Fracture of the diaphyseal region of the femur.	Yes (Stress fracture of the metatarsal bones)	No	Normal BMD value at the lumbar spine (52 years) Femur not evaluated	Normal	Normal	Normal	Normal	Low	Normal	Yes (17-year therapy with alendronate)
AFF20	c.7241G > A; p.Arg2414Gln (*FBN1*)	F	79	n.a.	Yes (Wrist fracture at 63 years. Fracture of finger and metacarpal bone)	Yes (Nephrolithiasis)	Osteoporosis at the lumbar spine and osteopenia at the femur neck and total femur (79 years)	Normal	n.a.	Normal	n.a.	Normal	n.a.	Yes (2-year therapy with alendronate)
AFF21	c.1331C > T; p.Thr444Ile (*SLC34A1*) c.1246G > A; p.Glu416Lys (*MEPE*)	F	67	n.a.	Yes (Fracture of ankle, metacarpal bone and metatarsal bone)	n.a.	Osteoporosis at the lumbar spine (67 years) Femur not evaluated	Normal	Normal	Normal	n.a.	Slightly low	n.a.	Yes (2-year therapy with alendronate, followed by 3-year therapy with ibandronate and 18-month therapy with denosumab)

(*continued*)

Table 1. Continued

Patient code	Identified rare genetic variant(s) (Gene)	Gender	Age at AFF manifestation	Characteristic of AFF(s)	History of fracture, other than AFF	History of nephrolithiasis and/or nephrocalcinosis	Osteoporosis or osteopenia (Age at DXA screening)	Serum activity of total ALP	Serum BALP value	Serum total calcium value	Urinary calcium value	Serum phosphate value	Urinary phosphate value	Anti-resorption therapy with bisphosphonates or denosumab before and/or at the time of AFF occurrence
AFF22	c.398C > T; p.Ala133Val (*SLC34A1*) c.818C > T; p.Thr273Met[a](*ALPL*)	F	90	n.a.	Yes (Atraumatic vertebral fracture of D11; ankle at the age of 40 years)	n.a.	Osteopenia at the lumbar spine (90 years) Femur not evaluated	Normal	n.a.	Normal	n.a.	Normal	n.a.	Yes (20-year therapy with ibandronate)
AFF23	c.1469C > T; p.Pro490Leu (*SLC34A1*)	F	89	n.a.	No	n.a.	Osteopenia at the lumbar spine, femur neck and total femur (90 years)	Normal	n.a.	Normal	n.a.	Normal	n.a.	Yes (10-year therapy with ibandronate)
AFF24	No rare genetic variant identified	F	72	n.a.	Yes (Traumatic fracture of the wrist at the age of 56 years)	n.a.	Normal BMD at the lumbar spine. Osteopenia at the femur neck and total femur (70 years)	Low	n.a.	Normal	n.a.	Normal	n.a.	Yes (5-year therapy with ibandronate followed by 2-year therapy of denosumab)
AFF25	c.722A > G; p.Lys241Arg (*SLC9A3R1*)	F	57	n.a.	Yes (Ankle at the age of 40 years. Proximal homerus at the age of 60 years)	Yes (Nephrocalcinosis)	Severe osteoporosis (T-score -3.1) at the lumbar spine and osteopenia at the femur neck and total femur (60 years)	Normal	n.a.	Normal	n.a.	Normal	n.a.	Yes (5-year therapy with ibandronate)

n.a., non-available; ALP, alkaline phosphatase; BALP, bone alkaline phosphatase; BMD, bone mineral density.
[a]Genetic variant identified in a previous study by Sanger sequencing of the *ALPL* gene.[9]

Waltham, MA, USA), quantified with Qubit BR assay (Thermo Fisher Scientific, Waltham, MA, USA), normalized according to quantification results, and then pooled and sequenced using MiSeq Reagent Kit v2 on MiSeq platform (Illumina, San Diego, CA, USA) by a "paired-end" protocol with 150 bp-length reads.

Raw reads were filtered using fastp (version 0.20.1) to remove adapters, low-quality reads and low-quality bases from the reads. The filtered reads were then mapped on the HG38 human reference genome using BWA-mem (version 0.7.17-r1188). PCR duplicate fragments were removed by MarkDuplicates, and base quality recalibration scores were computed by BaseRecalibrator (GATK version 4.1.1.0.). Single nucleotide variants and small intra-exonic insertions/deletions, were called using HaplotypeCaller and hard-filtered with VariantFiltration (GATK version 4.1.1.0.). To call large duplications or deletions on the genes of interest, a set of genomic regions that are stable for the number of copies was added to the capture panels. The ratio between the average coverage of each region of interest and the average coverage of the stable regions was computed on a set of samples to obtain the distribution of the ratios of each region. The outliers of each distribution were selected as deletion or duplication when the distance from the media was more than the established threshold.

The NGS analysis had analytic sensibility and specificity >99%, with a 10× coverage.

Variants were analyzed by VarSeq software (Golden Helix, Inc, Bozeman, MT, USA), using the Personal Genomics Variant Database (PGVD) v.1.0.25, and the databases reported in Table 2.

Annotated genetic variants were classified as "non-polymorphic" (rare variants) if they had a global minor allele frequency (GMAF) <0.01 in the general population, or as "polymorphic" (common variants) if they had a GMAF ≥0.01, according to GMAF value reported on ClinVar and/or in the NCBI Single Nucleotide Polymorphism database (dbSNP).

Missense variants were analyzed by "Polymorphism Phenotyping v2" (PolyPhen-2 v2.2.2r406), predicting possible functional effects of 1 amino acid substitution on a human protein, and classified as "benign", "possibly damaging", or "probably damaging".

Clinical pathogenicity of our identified variants was defined according to data reported in the ClinVar database, classifying them as VUS if they were reported with an uncertain clinical significance or with a conflicting interpretation of pathogenicity, or if they were not reported in ClinVar.

Table 2. Human genetic sources used for variant annotation.

Source	Version
Human gene variant databases	
Reference genome	GRCH38
ClinVar	2021-2011-04, NCBI
dbSNP	155, NCBI
RefSeq Genes	109 interim v2, NCBI
Variant allele frequency projects	
1000 Genomes	Phase3
ExAC Variant Frequencies	1.0
ESP/EVS	ESP6500SI-V2
GnomAD	2.0.1
HGMD Professional	2022.1

We classify a variant as "novel" if it is not currently reported in ClinVar, OMIM, Human Gene Mutation Database (HGMD), and/or published literature.

Results

NGS analysis confirmed the presence of the p.Thr273Met *ALPL* mutation in the AFF22 patient.

In 13 AFF cases (52.0%), no pathogenic or VUS rare variants were identified in the 76 screened genes. In the other 12 cases (48.0%), NGS identified 14 rare variants in 8 different genes. All these 14 identified variants were confirmed by Sanger sequencing.

Eight patients had a heterozygous rare variant in the *SLC34A1* or *SLC9A3R1* genes (32.0% of AFF analyzed cases), respectively, 4 cases bore a *SLC34A1* variant (16.0%) and 4 cases bore a *SLC9A3R1* variant (16.0%).

Other heterozygous rare variants were found in the *BMPR1B, CYP27B1*, *FBN1*, *MEPE, PIGO,* and *PHOSPHO1* genes, each in a single AFF case (4.0%).

The distribution of these 14 rare genetic variants in our AFF patients is reported in Table 1.

Of the 14 identified rare variants, only 8 (57.1%) are currently reported on ClinVar, all with conflicting interpretations of their pathogenicity or with uncertain clinical significance, and they can, thus, be classified as VUS. The other 6 rare variants are not either reported on ClinVar, OMIM and HGMD or published in scientific literature, and they can be, thus, classified as "novel".

None of the 14 identified rare variants in the *SLC34A1, SLC9A3R1*, *BMPR1B, CYP27B1*, *FBN1*, *MEPE, PIGO,* and *PHOSPHO1* genes were found in the 150 control cases.

The 14 identified rare genetic variants, their frequency in the general population, and their currently available clinical significance are reported in Table 3.

Discussion

Occurrence of AFF was reported in patients with some rare genetic metabolic bone disorders, often as a consequence of prolonged treatment with anti-resorption drugs.[4–8] The identification of rare gene variants responsible for altered mineral and bone metabolism and/or for impaired stress fracture healing, which may lead to atraumatic AFF, could be of importance in differential diagnosis with involutional osteoporosis, guiding the correct clinical and therapeutic management of patients and avoiding anti-resorption therapies to prevent future occurrence of AFF.

NGS screening of 76 genes involved in the regulation of the mineralization process, in 25 unrelated AFF cases, identified a relatively high frequency of heterozygous rare missense variants in the *SLC34A1* or *SLC9A3R1* genes.

Heterozygous germline mutations of the *SLC34A1* or *SLC9A3R1* genes have been respectively associated with autosomal dominant hypophosphatemic nephrolithiasis/osteoporosis types 1 and 2 (NPHLOP1 and NPHLOP2), two rare phenocopy diseases characterized by an excessive urinary loss of phosphate, leading to persistent hypophosphatemia, recurrent nephrolithiasis, bone demineralization, and osteoporosis.

The relatively elevated prevalence of heterozygous rare variants in the *SLC9A3R1* and *SLC34A1* genes in our AFF patients may suggest that variations of these two genes could

Table 3. Rare genetic variants identified in our AFF patients.

Gene (Encoded protein)	Role of the encoded protein	Variant	Exon	dbSNP number	MAF	ClinVar (n = number of entries)	OMIM	HGMD (Accession number)	Publications	PolyPhen-2 analysis
BMPR1B (BMPR1B)	BMPR1B positively regulates chondrocyte differentiation and endochondral ossification.	Heterozygote missense c.1366C > T; p.Arg456Trp	12	rs780280883	0.000040-0.00020[#] (T allele)	Uncertain significance ($n = 1$)	Not reported	Not reported	Not reported	Probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00).
CYP27B1 (CYP27B1)	CYP27B1 is the renal 1-alpha-hydroxylase that catalyzes the hydroxylation of 25(OH)-vitamin D to the 1,25-(OH)2 vitamin D.	**Heterozygote missense c.670G > T; p.Ala244Ser**	4	rs367743385	0.000008-0.00008[#] (T allele)	Not reported	Not reported	Not reported	Not reported	Possibly damaging with a score of 0.613 (sensitivity: 0.87; specificity: 0.91)
FBN1 (Fibrillin 1)	Fibrillin-1 is an extracellular matrix glycoprotein that serves as a structural component of calcium-binding microfibrils in elastic and non-elastic connective tissues. It was also demonstrated to regulate osteoblast maturation by controlling TGF-beta bioavailability and BMP levels and to negatively regulate osteoclastogenesis by binding and sequestering the osteoclast differentiation and activation factor TNFSF11.	Heterozygote missense c.7241G > A; p.Arg2414Gln	59	rs143863014	0.000019-0.0003[#] (A allele)	Conflicting interpretations of pathogenicity: Likely benign ($n = 1$); Uncertain significance ($n = 11$)	Not reported	Reported as possibly associated with Marfan syndrome. (CM062706)	Found in a male patient with sporadic Marfan syndrome[14].	Benign with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)
MEPE (MEPE)	MEPE is a calcium-binding phosphoglycoprotein secreted in the extracellular matrix and involved in the negative regulation of bone mineralization.	**Heterozygote missense c.1246G > A; p.Glu416Lys**	5	rs139465355	0.000041-0.00020[#] (A allele)	Not reported	Not reported	Not reported	Not reported	Probably damaging with a score of 0.995 (sensitivity: 0.68; specificity: 0.97)
PHOSPHO1 (PHOSPHO1)	PHOSPHO1 is phosphatase highly expressed in mineralizing regions of bone and in growth plates, that releases inorganic phosphate, initiating hydroxyapatite crystallization in the matrix vesicles for bone mineralization.	**Heterozygote missense c.142C > G; p.Arg48Gly**	3	rs564808415	0.00002-0.001[#] (G allele)	Not reported	Not reported	Not reported	Not reported	Possibly damaging with a score of 0.911 (sensitivity: 0.81; specificity: 0.94)

(*continued*)

Table 3. Continued

Gene (Encoded protein)	Role of the encoded protein	Variant	Exon	dbSNP number	MAF	ClinVar (*n* = number of entries)	OMIM	HGMD (Accession number)	Publications	PolyPhen-2 analysis
PIGO (PIGO)	PIGO is involved in the biosynthesis, in the endoplasmic reticulum, of glycosylphosphatidylinositol (GPI), a membrane anchor for numerous cell surface proteins.	Heterozygote missense c.626A > G; p.Asn209Ser	10	rs138028827	0.00040* (G allele)	Conflicting interpretations of pathogenicity: Likely benign (*n* = 1); Uncertain significance (*n* = 3)	Not reported	Not reported	Not reported	Probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00)
SLC34A1 (NPTIIa)	NPTIIa is a member of the type II sodium–phosphate cotransporter family, involved in actively transporting phosphate into cells via Na^+ cotransport in the renal brush border membrane and kidney phosphate reabsorption.	Heterozygote missense c.398C > T; p.Ala133Val	5	rs148976897	0.0020* (T allele)	Conflicting results on pathogenesis: Likely benign (*n* = 3), likely pathogenic for nephrocalcinosis (*n* = 1)	Not reported	Reported as associated with hypophosphatemic nephrolithiasis/ osteoporosis 1/ Fanconi syndrome. (CM067034)	Found in a male child with nephrocalcinosis, hypercalciuria and hypophosphatemia,[15] in two related children with nephrocalcinosis,[16] and in four unrelated Pakistani individuals with nephrolithiasis.[17]	Probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00)
SLC34A1 (NPTIIa)	NPTIIa is a member of the type II sodium–phosphate cotransporter family, involved in actively transporting phosphate into cells via Na^+ cotransport in the renal brush border membrane and kidney phosphate reabsorption.	Heterozygote missense c.782G > A; p.Arg261His	7	rs144306414	0.00060* (A allele)	Conflicting interpretations of pathogenicity: Benign (*n* = 1); Uncertain significance (*n* = 1)	Not reported	Not reported	Not reported	Possibly damaging with a score of 0.466 (sensitivity: 0.89; specificity: 0.90)
SLC34A1 (NPTIIa)	NPTIIa is a member of the type II sodium–phosphate cotransporter family, involved in actively transporting phosphate into cells via Na^+ cotransport in the renal brush border membrane and kidney phosphate reabsorption.	Heterozygote missense c.1331C > T; p.Thr444Ile	12	rs752434569	0.000016-0.000036# (T allele)	Not reported	Not reported	Not reported	Not reported	Possibly damaging with a score of 0.771 (sensitivity: 0.85; specificity: 0.92)
SLC34A1 (NPTIIa)	NPTIIa is a member of the type II sodium–phosphate cotransporter family, involved in actively transporting phosphate into cells via Na^+ cotransport in the renal brush border membrane and kidney phosphate reabsorption.	Heterozygote missense c.1469C > T; p.Pro490Leu	13	rs372577906	0.000017-0.00011# (T allele)	Conflicting interpretations of pathogenicity: Likely benign (*n* = 1); Uncertain significance (*n* = 1)	Not reported	Not reported	Not reported	Probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00)

(*continued*)

Table 3. Continued

Gene (Encoded protein)	Role of the encoded protein	Variant	Exon	dbSNP number	MAF	ClinVar (*n* = number of entries)	OMIM	HGMD (Accession number)	Publications	PolyPhen-2 analysis
SLC9A3R1 (NHERF1)	NHERF1 is a sodium/hydrogen exchanger regulatory cofactor that acts as organizer and regulator of transporters and channels at the apical side of epithelia through actin-binding ezrin–moesin–radixin proteins.	Heterozygote missense c.328C > G; p.Leu110Val	1	rs35910969	0.00599* (G allele)	Conflicting interpretations of pathogenicity: Benign (*n* = 2); Uncertain significance (*n* = 1)	Reported as associated with nephrolithiasis/ osteoporosis hypophosphatemic type 2	Reported as possibly associated with nephrolithiasis and bone demineralization. (CM085716)	Found in two unrelated patients both with impaired renal phosphate; one with recurrent nephrolithiasis and the other with severely reduced BMD.[18]	Benign with a score of 0.073 (sensitivity: 0.93; specificity: 0.84)
SLC9A3R1 (NHERF1)	NHERF1 is a sodium/hydrogen exchanger regulatory cofactor that acts as organizer and regulator of transporters and channels at the apical side of epithelia through actin-binding ezrin–moesin–radixin proteins.	Heterozygote missense c.458G > A; p.Arg153Gln	2	rs41282065	0.00080* (A allele)	Conflicting interpretations of pathogenicity: Likely benign (*n* = 1); Uncertain significance (*n* = 3)	Reported as associated with nephrolithiasis/ osteoporosis hypophosphatemic type 2	Reported as associated with nephrolithiasis and bone demineralization. (CM085715)	Mutation segregating with three family members with reduced renal phosphate reabsorption and recurrent nephrolithiasis.[18]	Probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00)
SLC9A3R1 (NHERF1)	NHERF1 is a sodium/hydrogen exchanger regulatory cofactor that acts as organizer and regulator of transporters and channels at the apical side of epithelia through actin-binding ezrin–moesin–radixin proteins.	**Heterozygote missense c.616T > C; p.Cys206Arg**	3	Not available	Not reported	Not reported	Not reported	Not reported	Not reported	Benign with a score of 0.098 (sensitivity: 0.93; specificity: 0.85)
SLC9A3R1 (NHERF1)	NHERF1 is a sodium/hydrogen exchanger regulatory cofactor that acts as organizer and regulator of transporters and channels at the apical side of epithelia through actin-binding ezrin–moesin–radixin proteins.	**Heterozygote missense c.722A > G; p.Lys241Arg**	3	rs373061379	0.000004-0.00009# (G allele)	Not reported	Not reported	Not reported	Not reported	Benign with a score of 0.001 (sensitivity: 0.99; specificity: 0.15)

MAF = minor allele frequency as reported as global MAF in ClinVar (indicated by *) or as MAF range of various studies in dbSNPs (indicated by #);
"Novel" variants are highlighted in bold.
NHERF1, sodium-hydrogen exchanger regulatory factor 1; NPTIIa, sodium/phosphate cotransporter 2A; BMPR1B, bone morphogenetic protein receptor type-1B; PIGO, phosphatidylinositol glycan anchor biosynthesis class O; CYP27B1, cytochrome P450 family 27 subfamily B member 1; BMP, bone morphogenetic protein; TNFSF11, tumor necrosis factor ligand superfamily member 11; MEPE, matrix extracellular phosphoglycoprotein; BMD, bone mineral density; OMIM, Online Mendelian Inheritance in Man®; HGMD, Human Gene Mutation Database.

represent a possible genetic risk factor for these fractures, especially in patients long-term treated with bisphosphonates and/or denosumab. Out of our 8 AFF patients with a *SLC34A1* or a *SLC9A3R1* variant, 5 had been receiving long-term therapy with bisphosphonates (62.5%) before and/or at the time of AFF occurrence, 2 did not receive medical therapy with either bisphosphonates or denosumab (both bearing a *SLC34A1* variant), and in one with a *SLC34A1* variant data about anti-resorption treatments were not available.

On the other hand, it could be suspected that the occurrence of AFF may represent a hitherto unsuspected clinical manifestation and/or an anti-resorption therapy-correlated adverse event in patients with NPHLOP disorders.

The *SLC34A1* gene encodes a member of the of the Type II sodium–phosphate cotransporter family, the NPTIIa protein, which is involved in actively transporting phosphate into cells via Na^+ cotransport, in the renal brush border membrane, playing a major role in phosphate homeostasis and bone mineralization.[19] The *SLC9A3R1* gene encodes the sodium–hydrogen exchanger regulatory factor 1 (NHERF1), a cytoplasmic scaffold protein that connects plasma membrane proteins with members of the ezrin/moesin/radixin family, helping to link them to the actin cytoskeleton and regulating their surface expression. This protein interacts with and regulates various proteins and major signaling pathways, including parathyroid hormone and the Wnt-β-catenin signaling. Moreover, the NHERF1 protein is implicated in transport regulation of NPTIIa to the apical membrane of the proximal tubular cells of kidneys, and in the control of its retrieval by parathyroid hormone,[20] showing a direct link between these two genes in the regulation of phosphate renal excretion.

Whole-exome sequencing in 51 families, whose members presented with at least 1 renal stone or with a renal ultrasound finding of nephrocalcinosis before the age of 25 years, identified dominant mutations of the *SLC9A3R1* gene, and recessive or dominant mutations in the *SLC34A1* gene, as disease-causing mutations of childhood nephrocalcinosis phenotype.[16] Data about history of renal diseases were available only for 4 out of our 8 AFF patients with *SLC9A3R1* or *SLC34A1* variants; of them, only 1 case (AFF25) was reported with nephrocalcinosis. Mutations of both the *SLC9A3R1* and *SLC34A1* genes have also been associated with autosomal dominant renal phosphate wasting.[21] Primary defect in phosphate metabolism, and derived hypophosphatemia, due to *SLC34A1* genetic variants or to defective NHERF1-derived altered NPTIIa transport on the renal cell membrane was shown to lead to secondary disturbances in calcium homeostasis (hypercalcemia and hypercalciuria),[22] induced by inappropriate elevated levels of serum 1,25-$(OH)_2$ Vitamin D_3. In our 8 AFF patients bearing 1 rare variant of the *SLC9A3R1* or *SLC34A1* genes, serum values of calcium and phosphate were available in 7 cases, all being normocalcemic, and 5 being normophosphatemic, one hyperphosphatemic (AFF8), and one with a mild hypophosphatemia (AFF21). Values of urinary excretion of calcium and phosphate were available only in 3 cases, all presenting normal calciuria and phosphaturia (AFF8, AFF9, and AFF12). Serum values of 1,25-$(OH)_2$ Vitamin D_3 was not assessed in any of our 25 AFF patients.

Out of the 4 different rare heterozygote missense variants of the *SLC9A3R1* gene identified in our AFF patients two (p.Leu110Val and p.Arg153Gln) were previously reported, respectively in 2 unrelated patients with impaired renal phosphate, one with recurrent nephrolithiasis and the other with severely reduced BMD, and in 3 family members with reduced renal phosphate reabsorption and recurrent nephrolithiasis.[18] No clinical data were available for our patient bearing the p.Leu110Val variant, except for the assessment of normal serum values of total ALP activity and calcium. Our patient bearing the p.Arg153Gln variant was a 24-year-old woman, at the time of this study, who had manifested an atraumatic AFF in the diaphyseal region of the right femur at the age of 4 years. No other fragility fractures were reported. She had no history of nephrolithiasis and presented normal levels of serum and urinary phosphate. DXA analyses, performed by the age of 7 years, showed osteoporosis of the lumbar spine, and osteopenia of the unfractured left femur neck and total femur.

Out of the 4 different rare heterozygote missense variants of the *SLC34A1* gene identified in our AFF patients only 1 (p.Ala133Val) was previously reported in 3 different studies, respectively, in a male child with nephrocalcinosis, hypercalciuria and hypophosphatemia,[15] in 2 related children with nephrocalcinosis,[16] and in 4 unrelated Pakistani individuals with nephrolithiasis,[17] indicating a strong association of this specific variant with a clinical phenotype characterized by renal calcifications. Data on history/presence of nephrolithiasis and/or nephrocalcinosis and on urinary excretion of calcium and phosphate were not available in our patient bearing the p.Ala133Val variant. The p.Ala133Val variant was functionally validated showing a defective phosphate uptake activity,[17] although not through a dominant-negative effect on the wild-type NPTIIa protein.

Heterozygous rare variants, all classified as VUS, of the *BMPR1B, CYP27B1, FBN1, MEPE, PIGO,* and *PHOSPHO1* genes were found in 6 different AFF patients. Currently, only mutations of the *BMPR1B, CYP27B1, FBN1* and *PIGO* genes have been associated with one or more clinical phenotypes, following a Mendelian inheritance, with mutations of the *CYP27B1* and *PIGO* genes having an autosomal recessive pattern of inheritance and mutations of the *BMPR1B* and *FBN1* genes showing an autosomal dominant pattern of inheritance. Heterozygote mutations of the *BMPR1B* gene have been associated with brachydactyly types A1, A2 and D.[23,24] Our patient bearing the *BMPR1B* variant did not manifest any malformation and/or anomalies of fingers, toes or limbs. The identified p.Arg456Trp variant has not been previously described as associated to any clinical phenotype and it is reported in ClinVar as of uncertain clinical significance. Heterozygote mutations of the *FBN1* gene have been associated with various rare clinical phenotypes. The extremely rare missense variant p.Arg2414Gln we identified in one of our AFF cases was previously reported as possibly associated with Marfan syndrome,[14] however our patient bearing this variant was not reported to have a Marfan phenotype or other clinical features typical of clinical phenotypes currently associated with *FBN1* gene mutations.

To date, the occurrence of AFFs, under therapy with bisphosphonates and/or in naïve patients, was identified in 7 monogenic rare bone metabolic diseases: hypophosphatasia (HPP), pycnodysostosis, osteopetrosis, X-linked hypophosphatemic rickets, X-linked osteoporosis, osteoporosis-pseudoglioma syndrome (OPPG), and osteogenesis imperfecta (OI).[4,5] Candidate gene studies, conducted to specifically search variants in few selected genes, identified mutations in one or more genes responsible for the aforementioned Mendelian disorders, in sporadic and familial cases of AFF.[4,5]

Interestingly, an exon-wide analysis performed on 13 unrelated women with AFF, using an array containing about 300 000 coding SNPs (including both synonymous and non-synonymous SNPs, variants in splicing sites and stop codons) and about 30 000 indels, demonstrated that AFF patients significantly accumulated two or more risk variants, compared to 268 unfractured women (controls).[25] Moreover, when the analysis was restricted to those variants likely to have damaging effects on protein function, authors found that over 80% of AFF had at least 1 variant with respect to only less than 10% of unfractured controls,[25] suggesting that the occurrence of these fractures is polygenic and AFF predisposition risk is associated with accumulation of variants in the coding regions of several genes. These findings could partially explain the relatively high frequency of one or more rare variants we found in our 25 AFF cases (48.0%).

Four whole-exome sequencing analyses[10,26–28] were performed, to date, in familial and/or unrelated cases of AFF, including both patients being under therapy with anti-resorption drugs and naïve patients, to investigate potential genetic risk factors for AFF occurrence.

A study by Roca-Ayats et al.[26] identified 37 coding variants in 34 different genes, shared among 3 sisters with AFF occurred under a 5-year treatment with bisphosphonates, consisting of 35 missense variants, one nonsense variant and one in-frame deletion. Then, authors screened these 34 genes for variants in 3 unrelated women with AFF, finding two additional variants in two cases, one in the *BRAT1* gene and one in the *CYP1A1* gene. None of all the 39 identified variants were found in 3 non-AFF controls. Out of the 34 mutated genes only two, the *FN1* gene, encoding fibronectin an extracellular matrix protein involved in positive regulation of correct mineralization, and the *GGPS1* gene, encoding the enzyme geranylgeranyl pyrophosphate synthase that is critical for osteoclast activity, were included in our multigenic panel, but no rare coding variants of these two genes were identified in our 25 AFF cases.

A second study[27] performed on 12 unrelated postmenopausal Caucasian women with AFF, compared to 4 women without AFF as control, found, after filtration and prioritization of rare variants predicted to be damaging and affecting genes shared among at least two AFF cases, a total of 272 variant in 132 different genes. Among the mutated genes only two were included in our mineralization panel, but none of them resulted to be mutated in our 25 AFF patients. The first one was the *LRP5* gene, encoding the homonym coreceptor that transduces Wnt signal, whose heterozygote mutations have been associated with osteosclerosis, osteopetrosis autosomal dominant Type 1, and endosteal hyperostosis, and homozygote or compound heterozygous mutations have been associated to OPPG. Interestingly, the second one was the *SCL34A3* gene that encodes the member C of Type II sodium–phosphate cotransporter family (NPTIIc), which, similarly to NPTIIa, is involved in the sodium-mediated cotransport of phosphate into the cells of the renal brush border, contributing to the maintenance of phosphate homeostasis. Variants of *SLC34A3* were identified in 2 of the 12 AFF cases (16.7%), a percentage comparable with the 16.0% of *SLC34A1* variants found in our AFF series. One deleterious variant in the *SLC34A3* associated to AFF was also reported in another study[28] that found 10 deleterious rare variants (9 missense and 1 frameshift variants) in 7 candidate genes (4 in *ENPP1*, 1 in *FGF23,* 1 in *CYP27B1*, 1 in *CYP3A4*, 1 in *SLC34A3*, 1 in *CYP2R1*, and 1 in *ALPL*) in 42 Japanese unrelated patients with AFF. All, but *CYP3A4,* these genes were included in our mineralization panel, but only *CYP27B1* was found to be mutated in one of our AFF patients. *CYP27B1* gene encodes the renal 1-alpha-hydroxylase that catalyzes the hydroxylation of 25(OH)-vitamin D to the 1,25-(OH)2 vitamin D. Biallelic inactivating mutations of the *CYP27B1* gene causes an inherited deficiency of the active form of vitamin D, and they have been associated with the vitamin D-dependent rickets Type 1A, while heterozygote mutations do not cause any Mendelian pathological phenotype.

A study of Zhou *et al.*[10] screened 3 AFF cases from two unrelated pedigrees, finding a rare variant in the *PLOD2* gene in the case from family 1 and a rare variant in the *TMEM25* gene in both the two cases from family 2. Unfractured members of the two families were not analyzed. Both *PLOD2* and *TMEM25* genes were not included in our mineralization panel.

In conclusion, the performance of NGS screening with our customized multigenic panel for mineralization found rare variants in various unsuspected genes that may have had a role in the development of AFFs, all but *CYP27B1* never identified before in AFF patients in the performed studied to date.

The relatively high prevalence of rare VUS of the *SLC9A3R1* and *SLC34A1* genes prompts to extend the genetic study to a larger number of AFF cases to confirm the obtained data, preferably in the context of prospective studies, by collecting clinical data and detailed clinical and family history.

Future studies should address the link between the identified VUS mutations within families of the AFF cases, as well as the functionality of mutated proteins by *in vitro* studies.

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Supplementary material

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Data availability

The data set analyzed in the current study is not publicly available but is available from the corresponding author upon reasonable request.

Statement of human subject research

This study was approved by the Review Board of the "Area Vasta Centro, Regione Toscana" at the "Azienda Ospedaliera-Universitaria Careggi" (Rif. CEAVC10601_oss). Patients signed an informal consent form before inclusion in this study, after receiving a full explanation of the purpose and nature of the study. The study was performed according to the Declaration of Helsinki.

Clinical data were retrospectively retrieved from medical records, and blood and DNA samples, which were collected and analyzed anonymously; each enrolled patient was identified only by an anonymous unique alphanumeric code during the entire study, and in the writing of this paper.

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