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A gain of function mutation in *TNFRSF11B* causes osteoarthritis with chondrocalcinosis
A gain of function mutation in \textit{TNFRSF11B} causes osteoarthritis with chondrocalcinosis

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**ABSTRACT**

**Objective** To identify pathogenic mutations that reveal underlying biological mechanisms driving OA.

**Methods** Exome sequencing was applied to two distant family members with dominantly inherited early onset primary osteoarthritis at multiple joint sites with chondrocalcinosis (FOA). Confirmation of mutations occurred by genotyping, and linkage analyses across the extended family. The functional effect of the mutation was investigated by means of a cell-based assay. To explore generalizability, mRNA expression analysis of the relevant genes in the discovered pathway was explored in preserved and osteoarthritic articular cartilage of independent patients undergoing joint replacement surgery.

**Results** We identified a heterozygous, probably damaging, read-through mutation (c.1205A=>T; p.Stop402Leu) in \textit{TNFRSF11B} encoding osteoprotegerin that is likely causal to the osteoarthritis phenotype in the extended family. In a bone resorption assay, the mutant form of osteoprotegerin showed enhanced capacity to inhibit osteoclastogenesis and bone resorption. Expression analyses in preserved and affected articular cartilage of independent osteoarthritis patients showed that up-regulation of \textit{TNFRSF11B} is a general phenomenon in the pathophysiological process.

**Conclusions** Albeit that the role of the molecular pathway of osteoprotegerin has been studied in osteoarthritis, we are the first to demonstrate that enhanced osteoprotegerin function could be a directly underlying cause. We advocate that agents counteracting the function of osteoprotegerin could comply with new therapeutic interventions of osteoarthritis.

**Keywords:** osteoarthritis, RANK-antagonism, \textit{TNFRSF11B}, osteoclastogenesis, exome sequencing
INTRODUCTION

Osteoarthritis (OA) is a prevalent, complex, disabling disease of the articular joints, characterized by degradation of the hyaline articular cartilage and remodeling of the subchondral bone with sclerosis. As the population ages and obesity rates rise, OA is becoming more prevalent and current estimates of men and women aged over 60 years having symptomatic OA are 9.6% and 18.0%, respectively.[1] Yet, there is no effective therapy to reverse or slow down the disease, analgesia, physiotherapy and, at end stage, joint replacement surgery are the main treatment options. As a result, osteoarthritis has a large detrimental impact on the quality of life of the elderly and causes a major burden on health, and social care systems, with indirect costs being predominant.[1, 2] To allow the development of new therapies, there is an ongoing need for insight into the underlying biological mechanisms driving OA. Despite the detection of compelling OA susceptibility loci[3, 4], such insights, until now, remain limited. To detect rare mutations with large to moderate effect sizes, recently focus has been to apply next generation sequencing of exomes of severely affected family-based patients. The value of identified rare variants lies in the characterization of causal gene functions and underlying novel pathways in complex disease processes.[5, 6] Notably in this respect; among the identified genes with mutations causing early-onset families with OA associated syndromic skeletal phenotypes such as collagen type II (COL2A1),[7] the growth differentiating factor 5 (GDF5),[8] collagen type 11-alpha-1 (COL11A1),[9, 10] cartilage oligomeric protein (COMP)[11] and extracellular signaling molecule and member of the TGF-β superfamily (SMAD3)[12, 13] were three genes that appeared to additionally confer risk to common OA phenotypes, namely SMAD3,[15] COL11A1,[16, 17] and the GDF5 gene which reached genome wide significance.[8, 14]

Previously, we reported on a single early onset family with familial generalized osteoarthritis (FOA) without any dysplasia, with radiographic chondrocalcinosis as reflected by calcifications within the joint- and fibro-cartilage.[18] Candidate gene screening, showed that none of the genes encoding the extracellular matrix structural proteins were involved in the pathogenesis of this osteoarthritis phenotype.[18] Furthermore, a genetic linkage search in this and 6 other FOA families revealed significant linkage on chromosome 2q32 albeit that the attribution of this particular family to the linkage was moderate and Sanger sequencing of positional exons failed to definitively indicate the causal mutation.[19] Recently, a relevant extension was established in the ascertainment of the family under study; additionally affected distant family members with the phenotype were detected whereas third generation family members definitively developed the FOA phenotype. Such extensions increases the power of successful identification of genes considerably. To identify pathogenic osteoarthritis mutations, we applied exome sequencing to two distant family members.

MATERIALS AND METHODS

Study design. To identify pathogenic mutations underlying development of OA exome sequencing was performed for 2 distantly related FOA family members. After applying an eligible prioritization scheme five candidates were selected for de novo
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Genotyping across the extended family (N=16). Linkage analysis was performed for the mutation that was shared among all affected members and the mutation was validated by de novo genotyping of independent OA cases (N=1,467) and healthy controls (N=744). Functionality of the mutation was investigated in an in vitro cell-based model, and generalizability was investigated by comparing gene expression in OA affected cartilage with preserved cartilage (N=33).

An overview of the workflow for the analyses, identification of the causal mutant, and follow-up on the mutation is provided in Supplementary Figure 1.

Study populations
Cases: FOA family members. Previously, we reported on an early onset family with familial generalized osteoarthritis (FOA MIM 165720·1) without any dysplasia, and with radiographic chondrocalcinosis as reflected by calcifications within the joint- and fibrocartilage (Figure 1A and Supplementary Table 1).[18] Family members were collected through Rheumatology clinics from different parts of the Netherlands and France. The age of onset of osteoarthritis in the family varies between 30 and 50 years and osteoarthritis arises in the absence of mild or severe chondrodysplasia. The phenotype within these families is characterized by distinct progressive osteoarthritis with symptoms and radiographic characteristics of osteoarthritis at multiple joint sites simultaneously, including involvement of the hands with noduli, knees, hips, and spine. More recently, relevant additional family members were ascertained (Figure 1B, subject 4, 5, and 16) with the unique FOA phenotype of this family. The study was approved by the medical ethic committee and written informed consent was obtained from all participants.

Controls: Occurrence of the OPG read-through mutation in the population was tested by de novo genotyping in healthy subjects and OA cases. Middle-aged partners (N=744) of the offspring of nonagenarian siblings from the Leiden Longevity study (LLS)[20] were considered as controls for OA cases and are further named ‘random controls’. OA cases included were selected from the Genetics osteoarthritis and Progression (GARP) study (N=177),[21] the Patients Prospectively Recruited in Knee and Hip Arthroplasty (PAPRIKA) study performed at the Leiden University Medical Center (Dept. Orthopedics; N=1,137),[22] and the ongoing Research Arthritis and Articular Cartilage (RAAK) study (N=153) aimed at the biobanking of joint materials (cartilage, bone and where available ligaments) and bone marrow derived mesenchymal stem cells (hip joints only) of OA patients and controls in the Leiden University Medical Center and collaborating outpatient clinics in the Leiden area. More detailed description of the studies included can be found in the Supplementary methods.

Ethical permission for all studies described was obtained from the appropriate medical ethical committee. Written informed consent was obtained from all participants following detailed explanation of the study.

Exome sequencing. Exome sequencing of the FOA family members was performed by Illumina HiSeq2000 technology (Beijing
Genotypeing. Heterozygous deleterious gene variants shared among the two family members were chosen to fit efficiently in a Sequenom multiplex assay designed by the Assay Designer software version 3.1 (Sequenom, San Diego, CA). Single nucleotide polymorphisms (SNPs) were genotyped by mass spectrometry (the homogeneous MassARRAY system; Sequenom, San Diego, CA) using standard conditions and as described elsewhere.[24] In short, PCR reactions were carried out in a final volume of 5 µl and contained 2.5 ng of genomic DNA. Genotypes were assigned by using Genotyper version 3.0 software (Sequenom, San Diego, CA). Variants were genotyped across available FOA members (Supplementary Table 1 and Supplementary Table 3), osteoarthritis case studies (GARP, PAPRIKA study and RAAK), and random controls (LLS).

Linkage analysis. To assess the logarithm of the odds (LOD) score of the mutation in TNFRSF11B within the extended family, two point model-based linkage analysis was performed using the FASTLINK 2.2 version of the linkage program MLINK.[25] The disease locus was modeled as an autosomal-dominant trait with a disease frequency of 0.001, as symptomatic and radiographic osteoarthritis at multiple joint sites before the age of 50 years is rare. The TNFRSF11B allele frequency was set at 0.01. To perform a conservative analysis penetrance were assumed to be 100% among definitively phenotyped individuals (Patient 1, 2, 3, 4, 5, 6, 7, 8, 12, 14, and 16, Figure 1, and Supplementary Table 1). Family members with uncertain phenotype were considered unknown (Patient 9, 10, 13, and 15, in Figure 1 and Supplementary Table 1).

In vitro mutagenesis. A mammalian expression vector (pORF9) containing the human TNFRSF11B open reading frame was obtained from InvivoGen. The point mutation causing read-through was introduced using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies Inc.) according to the manufacturer’s protocol with the following primers: Forward 5’-TAAGCTGCTTATCATGAAAAATGGCCATTGAGC-3’; Reverse 5’-GCTCAATGGCCATTTCCAGTAGATAAGCAGCTTA-3’. Introduction of the aimed mutation and exclusion of additional mutations by PCR artifacts was confirmed by sequencing from both strands.
Production of recombinant osteoprotegerin and Western blot analysis. For the production of recombinant osteoprotegerin (OPG), HEK293T cells were cultured in OPTI-MEM I-Reduced Serum Medium (Invitrogen). Cells were transfected either with empty vector or vector with wild-type or mutant TNFRSF11B using FuGENE 6 reagent (Roche). Tissue culture supernatants were collected after three days, cleared of debris by centrifugation, and stored frozen. To confirm expression and quality of the recombinant proteins in the conditioned media, Western blotting was performed on 45 µl tissue culture supernatant as described previously [26] using the rabbit monoclonal antibody EPR3592 (Epitomics; 1:5000 diluted) to detect OPG. Subsequently, concentration of OPG in the tissue culture supernatant was determined by ELISA (R&D Systems) following the manufacturer’s instructions.

In vitro osteoclastogenesis. Putative difference in the activity of wild-type and mutant OPG was investigated by applying an in vitro model of osteoclastogenesis on 96-wells plates with pieces of human bone. Poietics™ human osteoclast precursors (Lonza) were seeded onto the OsteoAssay Human Bone Plate (Lonza; 10⁴ cells per well) in the absence (negative control) or in the presence (positive control) of RANKL. Either nothing, or equal volumes of tissue culture supernatant from the HEK293T cells was added to osteoclast growth and differentiation medium (Lonza) to a final concentration of 100ng/ml OPG. Osteoclast precursors were cultured for 6 days before adding fresh osteoclast growth and differentiation medium. Subsequently, cells were grown for 16-18 hours and media were harvested for analysis.

To analyze osteoclastogenesis, tartrate-resistant acid phosphatase staining (TRAP; Takara Bio Inc.) at day 7 of poietics human pre-osteoclast cultures was carried out following the manufacturer’s protocol. Concentration of CTX-I in the media was determined by applying CTX-I CrossLaps® enzyme-linked immunosorbent assays (ELISAs; IDS Ltd.) according to the manufacturer’s protocol. The amount of CTX-I released in the tissue culture supernatant reflects the amount of bone resorption by mature osteoclasts. The in vitro osteoclastogenesis assays were carried out 2 times in triplicate.

Gene expression analysis. To explore the generalizability of the discovered pathway towards common forms of osteoarthritis, mRNA expression was analyzed in an available dataset (Illumina HT-12 v3 microarray) of 33 paired preserved and osteoarthritic cartilage samples of the RAAK study. Following RNA isolation of the collected cartilage, samples were prepared for gene expression by microarray analysis as described in detail in the Supplementary methods. Subsequently, data were exported for analyses using Limma.[27] As implemented in Limma, a paired t-test was applied to all sample pairs. Overall mean normalized probe expression levels of the measured genes in cartilage ranged from 6.58 to 14.91 with a mean of 7.4 and a median of 7.1 with a strong right tailing.
RESULTS

Identification of read-through mutation in *TNFRSF11B*. Whole exome sequencing was applied to two distant FOA family members (Figure 1B; subject 1 and 4) and identified 57,018 and 60,652 variants, respectively, which fulfilled the quality criteria in each patient. To identify pathogenic mutations a prioritization scheme was applied in which we excluded variants present in dbSNPv132 (including 1000 Genomes Pilot project data), synonymous variants, tolerated missense variants as determined by SIFT (http://sift.jcvi.org/), intergenic variants and intron variants. In addition, we excluded variants detected in in-house analyzed human genomes (N=221), in-house analyzed human exomes (N=43), and analyzed independent human genomes by the Genome of the Netherlands project (GoNL. N=473; www.nlgenome.nl).[28] This reduced the number of variants to, respectively, 102 and 105 variants per subject that were predicted to affect gene function (see Supplementary Table 2). Given that these two FOA patients were distantly related (Figure 1B subject 1 and 4; six meioses apart) they are expected to share the causal variant. We found in total five heterozygous deleterious gene variants that were shared among the two patients: 3 novel missense damaging, 1 splice site mutation, and 1 read-through mutation located in, respectively, the preferentially expressed antigen in melanoma family member 22 (*PRAMEF22*), dynein axonemal heavy chain 10 (*DNAH10*), tetraspanin 8 (*TSPAN8*), mitotic control homolog gene (*DIS3*), and the tumor necrosis factor receptor superfAMILY member 11b (*TNFRSF11B*; Table 1).

Genotyping of these variants in all...
Table 1. Detected novel variants overlapping in family member 1 and 4. * Variants are novel, missense damaging, splice-sites or read-through, not present in dbSNPv132 (including 1000 Genomes Pilot project data), and not detected in in-house analyzed human genomes (ILS, N=221), in-house analyzed human exomes (GARP, N=43) and analyzed human genomes by means of the Genome of the Netherlands Project (GoNL, N=473).

Legend: CHR: chromosome; POS: chromosomal position; Base: base change; codon: codon change; AA subst.: amino acid substitution; Expr: relative expression level as measured in the microarray analyses of 33 osteoarthritic and preserved paired cartilage samples; p: nominal P-value of the differential expression between paired osteoarthritic and preserved samples (N=33 pairs); ND: not determined.

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<th>Codon*</th>
<th>AA subst</th>
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<td>119936614</td>
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<td>TAA=&gt;TTA*</td>
<td><em>=&gt;L</em></td>
<td>Tumor necrosis factor receptor superfamily, member 11b (TNFRSF11B)</td>
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The re-read-through mutation on OPG protein function. OPG is a soluble decoy receptor which inhibits osteoclastogenesis by competing with the receptor activator of the nuclear factor κB ligand (RANKL) for the binding of nuclear factor κB (NFκB) on the membrane of pre-osteoclasts. The read-through mutation on OPG protein function was investigated in an in vitro osteoclastogenesis assay. Mutant OPG gene was generated by site directed mutagenesis and was expressed in HEK293T cells. Protein integrity was confirmed by Western blotting. Recombinant wild-type and mutant OPG proteins were produced in transfected HEK293T cells. Recombinant OPG was added to osteocalcin-dependent osteoclastogenesis assay. The effect of the (X402L) read-through mutation on OPG protein function was investigated in an in vitro osteoclastogenesis assay. Western blotting was used to confirm protein integrity. Recombinant proteins were added to osteocalcin-dependent osteoclastogenesis assay. Osteoclast formation was monitored and compared to wild-type OPG.
OPG mutation directly causal to osteoarthritis

differentiating poietics human pre-osteoclasts seeded onto OsteoAssay™ Human Bone Plates. After 1 week, efficiency of bone resorption upon osteoclastogenesis of the osteoclast precursors was quantified by determining the amount of CTX-I released in the medium of the differentiating osteoclasts (Figure 2A). A clear difference could be observed between cells cultured in the presence or in the absence of OPG. Figure 2 shows that the addition of RANKL to pre-osteoclasts greatly enhanced osteoclastogenesis as can be concluded from the higher concentrations of CTX-I (Figure 2A, +). Adding medium from HEK293T cells transfected with empty vector did not have an effect (Figure 2A, ‘+ sup’). However, addition of recombinant wild-type OPG (Figure 2A, ‘+ wt OPG’) or mutant OPG (Figure 2A, ‘+ mt OPG’) significantly reduced bone resorption, respectively, 1.2-fold ($p=0.049$) and 1.6-fold ($p=8.4\times10^{-5}$) reflecting a statistical significant increased capacity of the mutant-OPG to inhibit osteoclastogenesis as compared to the wild type ($p=4.7\times10^{-3}$). To visually inspect the osteoclastogenesis in this experiment

Generalizability of the OPG/RANK/RANKL pathway in osteoarthritis. To investigate the a tartrate-resistant acid phosphatase (TRAP) staining was performed for the different conditions. Mature osteoclasts are distinguished as large multinucleated TRAP positive cells (Figure 2B, indicated with an arrow). Together, these data demonstrate that the identified read-through mutation in TNFRSF11B, causing the FOA phenotype, enhances OPG protein function response of TNFRSF11B and the tumor necrosis factor superfamily member 11 (TNFSF11) gene, encoding RANKL, to the general osteoarthritis pathophysiological process in articular cartilage, we explored a microarray expression dataset of osteoarthritis affected and preserved cartilage, taken from the same joints, of 33 independent osteoarthritis patients that underwent joint replacement due to end stage osteoarthritis disease (RAAK study). TNFRSF11A encoding RANK appeared to be expressed at a very low level (in the lowest quartile of expression) and was therefore not detected well. In contrast, the expression levels of TNFRSF11B in osteoarthritic and preserved cartilage samples appeared in the highest expression quartile with little inter-individual variation. A highly significant up-regulation of both TNFRSF11B (2.1-fold change, $p=1.9\times10^{-8}$) and TNFSF11 (1.2-fold change, $p=3.1\times10^{-6}$) was observed in osteoarthritic cartilage as compared to preserved cartilage of the same joint, indicating that these genes are responsive to the OA process in articular cartilage (Supplementary Table 5).

It has been shown that bone homeostasis merely depends on the ratio between TNFRSF11B and TNFSF11.[29] Therefore, we compared this ratio in both, osteoarthritic and preserved cartilage. As shown in Figure 3, we found a significant increase of the TNFRSF11B/TNFSF11 ratio ($p=2.0\times10^{-4}$) in osteoarthritic articular cartilage which may contribute to respective mineralization of the cartilage and eventually formation of bone, a major hallmark of the ongoing osteoarthritis disease process.[30]

DISCUSSION

We have identified a heterozygous read-through mutation (c.1205A=>$T$; p.Stop402
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Figure 2. Read-through mutation in the \textit{TNFRSF11B} gene results in gain of OPG function.

A. Levels of CTX-I were measured in the medium of the assay. Results shown are the average of two independent assays performed in triplicate ($^* p < 0.05$ $^** p < 0.005$ $^*** p < 0.0001$ ANOVA). Legend: ‘-’: nothing added; ‘+’: RANKL added, ‘+ sup’: RANKL and medium from HEK293T cells transfected with empty vector added; ‘+ wt OPG’: RANKL and recombinant wild-type OPG added; ‘+ mt OPG’: RANKL and recombinant mutant OPG added. Added OPG was present in the tissue culture supernatant at a concentration of 100 ng/ml. B. Representative example of histochemical staining for Tartrate-resistant acid phosphatase (TRAP) at day 7 of poietics human pre-osteoclast cultures on the OsteoAssay\textsuperscript{TM} Human Bone Plate. Active and fully differentiated osteoclasts are detected as large multinucleate cells (generally accepted standard for mature osteoclasts is presence of 3 or more nuclei per cell) which are characterized by high TRAP expression (visualized as light purple; white arrows indicate some of the osteoclasts shown in the pictures and black arrowhead points at an example of a bone slice).

Leu) in \textit{TNFRSF11B} that is likely causal to the severe osteoarthritis phenotype in the here studied extended FOA family. Functional analyses showed that this mutation establishes a gain of function of the encoding protein OPG. Given that the mutant OPG decoy receptor more efficiently antagonizes osteoclastogenesis and the FOA phenotype is characterized by calcifications within the joint- and fibro-cartilage, we hypothesize that the detected mutation acts via an unfavorable interplay between (subchondral) bone and cartilage towards enhanced matrix mineralization. Gene expression analyses of preserved and osteoarthritic articular cartilage of independent OA patients showed that enhanced OPG-mediated antagonism is a general phenomenon of the pathophysiological process within osteoarthritic articular cartilage. This is in line with other reports showing increased expression of OPG in arthritic cartilage compared to healthy cartilage.[31, 32] Also during intervertebral disc degeneration, a process highly comparable to the pathological processes occurring in articular cartilage during OA, OPG appeared to be increased in correlation with degeneration grade and coinciding with the presence of microscopic calcifications.[33] Albeit that the role of the molecular pathway of OPG has
been studied in osteoarthritis, a direct causal role towards matrix mineralization has not been reported up until now and it may provide new clues to the development of anti-osteoarthritic drugs.

Retrospectively, we observed in our family a positional linkage signal at the position of the TNFRSF11B gene (Supplementary Table 3).[19, 34] Additionally, of note is a report of Baldwin et al. that indicated linkage to chromosome 8q in a family with early onset osteoarthritis and chondrocalcinosis at a locus encompassing the TNFRSF11B gene.[35] Given the results, it would be interesting to further investigate the status of the TNFRSF11B gene of the reported family by next generation sequencing. Although TNFRSF11B was not previously detected as OA susceptibility gene by virtue of genome wide association studies, consistent genetic associations of TNFRSF11B with knee OA were found in the general population,[32, 36] in particular in association with OA progression as measured by changes in osteophyte grade. More investigations are necessary to explore whether genetic variations in genes of the OPG pathway confer risk to common OA.

Transgenic mice that overexpress TNFRSF11B had increased skeletal radiodensity, osteopetrosis, and increased bone density due to decreased numbers of mature osteoclasts,[37] however, no reference or investigation has been made to osteoarthritis as being part of the phenotypes of these mice or, for that matter, radiographic chondrocalcinosis. Since long, epidemiological studies have shown that high bone mineral density is associated with development of OA[38, 39] and may also affect OA progression.[40] Therefore, alternatively to the mentioned enhanced matrix mineralization of the cartilage, expression of mutant OPG could result in systemic higher bone mass thereby predisposing to OA due to a suboptimal cartilage-bone interplay. Since we have no bone density measurements available of our FOA family members, the role of systemic high bone density in the osteoarthritis onset requires further investigation. In contrast, loss of function mutations in the TNFRSF11B gene, that abolish the inhibitory action on osteoclastogenesis, have been found to cause Juvenile Paget’s disease characterized by accelerated bone turnover, fractures of the long bone and hyperphosphatasia.[41] Furthermore, SNPs within the TNFRSF11B gene have been found to be associated in genome wide association studies to common osteoporosis or low bone mineral density.[42]
Despite the fact that it has been convincingly demonstrated that a strong interaction between subchondral bone and the articular cartilage exists and that changes in mineralization of both bone and cartilage reflect pathophysiology of osteoarthritis, several seemingly contradictory observations have been made and the direction of the changes of the subchondral bone density and mineralization in osteoarthritis patients appeared unclear and possibly dependable on the disease status.\cite{43, 44} More recently, osteoarthritis patients were shown to benefit from treatment with strontium ranelate, which is a drug licensed for osteoporosis and acts by increasing bone formation while decreasing bone resorption.\cite{45} These studies, however, have been subject of debate\cite{46, 47} and seem in contrast with our current findings and with the extensive literature indicating that individuals with high systemic bone mass are at increased risk for the incidence of osteoarthritis.\cite{48} It could be speculated that, initially, strontium ranelate enhances stabilization of the dynamically changing OPG/RANKL ratios as it was observed across different grades of OA,\cite{49} resulting in OPG and RANKL levels in the joint balancing towards a net ratio of bone formation. This may temporarily strengthening the joint and decelerate OA progression. Although therapeutic treatment of rats with another bone-forming agent, OPG-Fc, did not directly improve the OA phenotype, it did decrease pain in particular when starting the treatment at early stages.\cite{50} Increased bone thickness as a result of the treatment may prevent enervation of the joint leading to a ‘desensitization’. Careful investigation of the long term effects of these bone forming therapies in osteoarthritis is, definitely, necessary\cite{30} thereby taking the existence of different (sub)types of OA characterized by differences in bone mineral density\cite{51} into account.

The molecular pathway of the OPG/RANKL in terms of its manipulation for therapeutic benefit has been studied extensively, especially to enhance the OPG-mediated RANK antagonism in osteoporosis, or, in the case of cancer, growth of bone metastasis.\cite{52} These studies showed that all of the C-terminal domains of OPG are necessary for high affinity association with RANKL.\cite{53} As a result, the design of short recombinant, monomeric OPG molecules that mimic both the receptor binding and dimerization functions of the multi-domain, 100-kDa homodimeric OPG polypeptide has been indicated as challenging.\cite{52} Given that the here detected read-through mutation results in the addition of 19 amino acids at the C-terminus of the OPG protein, we hypothesize that the gain of function may be established through an enhanced stability of OPG dimers thereby increasing the efficiency of RANKL binding. The use of the here identified X402L mutant OPG protein could provide a putative new starting point to further establish such a stable molecule.

In summary, we have identified a read-through gain of function mutation within the TNFRSF11B gene that is likely causal to the FOA phenotype in the here studied family. With this result, we are the first to demonstrate that enhanced OPG mediated RANK antagonism could be directly underlying the onset of osteoarthritis. The exact mechanism on how this is brought about remains to be established. Given that enhanced OPG-mediated antagonism appears to be a more general phenomenon in
the pathophysiological process of osteoarthritic cartilage, we advocate that, in contrast to the use of bone-forming therapies, agents counteracting OPG function could comply with the development of new disease modifying treatments in osteoarthritis.

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Author Contributions: YR, ES and IM contributed to the overall study design, data interpretation and writing of the manuscript. YR performed the functional studies. RB contributed to DNA sample preparations and confirmation of exome sequencing findings by de novo genotyping. SB contributed the mRNA expression analyses. MK and RN contributed to collection and interpretation of clinical data and cartilage samples. KY and EWL contributed to the bio-informatics of the exome sequencing data. All authors read and approved the manuscript.

Competing Interest: Authors have no conflict of interest to declare.

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OPG mutation directly causal to osteoarthritis

Supplementary methods

STUDIES:

Controls

Leiden Longevity Study (LLS). In the current study, the OPG read-through mutation has been tested for presence among the partners (N=744) of the offspring LLS referred to as random controls. Families were eligible to participate in the LLS study when at least two long-lived siblings were alive and over 89 years old for males and over 91 years for females, representing less than 0.5% of the Dutch population in 2001 (so-called ‘nonagenarians’).[3]

Cases

Genetics osteoarthritis and Progression (GARP). The GARP study consists of 192 sibling pairs concordant for clinical and radiographically (K/L score) confirmed osteoarthritis at two or more joint sites among hand, spine (cervical or lumbar), knee or hip. Conventional radiographs of the hands (dorso-volar), knees (posterior-anterior (PA) in weight bearing semiflexed and lateral), hips (PA), lumbar (PA and lateral), and cervical spine (anterior-posterior, lateral, and transbuccal) were obtained from all participants. Detailed description of the GARP study has been published previously.[4] Occurrence of the OPG read-through mutation among OA cases was tested by de novo genotyping of 177 cases from the GARP study.

Patients Prospectively Recruited in Knee and Hip Arthroplasty (PAPRIKA). The Paprika study is performed at the Leiden University Medical Center (Dept. Orthopedics) and consists in a long-term follow-up study of patients that have undergone total joint replacement (TJR) at hip or knee.[5] Patients of Caucasian descent were included when they were diagnosed with primary osteoarthritis based on radiographs and the ACR rheumatology classification criteria. Patients with secondary osteoarthritis or patients requiring a revision were excluded from the PAPRIKA study. For the study described here we genotyped the detected mutations in 1137 patients of the PAPRIKA study.

Research Arthritis and Articular Cartilage (RAAK). The RAAK study is aimed at the biobanking of joint materials (cartilage, bone and where available ligaments) and bone marrow derived mesenchymal stem cells (hip joints only) of OA patients and controls in the Leiden University Medical Center and collaborating outpatient clinics in the Leiden area. In the current study we genotyped the detected mutations in 153 RAAK patients, and determined gene expression in 61 paired samples of osteoarthritic and macroscopically unaffected cartilage.

Gene expression analysis

To explore the generalizability of the discovered pathway towards common forms of osteoarthritis, mRNA expression was analyzed in an available dataset (Illumina HT-12 v3 microarray) of 33 paired preserved and osteoarthritic cartilage samples of the RAAK study.

Cartilage collection. Within the RAAK study we collected paired preserved and OA affected cartilage samples from Caucasian end stage OA patients undergoing joint replacement surgery for primary OA in the Leiden University Medical Centre. At the moment of collection (within
2 hours following surgery) tissue was washed extensively with phosphate buffered saline (PBS) to decrease the risk of contamination by blood, and cartilage was collected of the weight-baring area of the joint. Cartilage was classified macroscopically and collected separately for OA affected and preserved regions (classification was done according to predefined features for OA-related damage based on color/whiteness of the cartilage, based on surface integrity as determined by visible fibrillation/crack formation, and based on depth and hardness of the cartilage upon sampling with a scalpel). During collection with a scalpel, care was taken to avoid contamination with bone or synovium. Collected cartilage was snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

**RNA isolation.** Cartilage samples were pulverized using a Retsch MM200 under cryogenic conditions. On average 150 mg of pulverized cartilage was dissolved in 1 ml of Trizol reagent, and mixed vigorously. After addition of 200μl of chloroform the sample was mixed and centrifuged for 15 minutes at 16,000g. The clear aqueous layer was transferred to a new vial and 1 volume of 70% ethanol/DEPC-treated water was added to precipitate RNA. The RNA was collected using Qiagen mini columns according to the manufacturers protocol. The RNA quality was assessed using a Bioanalyzer lab-on-a-chip and quantity was assessed using a nanodrop spectrophotometer. RIN values above eight were considered suitable for gene expression analysis.

**Microarray expression.** 0.5 μg of total RNA was used as template for first- and second-strand reverse transcription steps using an Ambion (Life Technologies Europe BV, Rijswijk, The Netherlands) RNA amplification kit. After in vitro transcription, amplification, and labeling with biotin-labeled nucleotides Illumina HT-12 v3 microarrays were hybridized (Illumina, San Diego). Sample pairs were randomly dispersed over the microarrays, however each pair was measured on a single chip of in total eight chips. The microarrays were read using an Illumina Beadarray 500GX and after basic quality checks using Beadstudio the intensity values were normalized using the “rsn” option in the Lumi R-package. The corresponding signals increase exponentially with relative expression probe-levels. Indicated units are light intensity as provided by Illumina. The subsequently obtained raw probe-level data were exported for analyses using Limma.[6] As implemented in Limma, a paired t-test was applied to all sample pairs. Overall mean normalized probe expression levels of the measured genes in cartilage ranged from 6.58 to 14.91 with a mean of 7.4 and a median of 7.1 with a strong right tailing.

**References**

OPG mutation directly causal to osteoarthritis


Supplementary Table 1. Radiographic and clinical abnormalities in members of a family with autosomal dominant FOA with radiographic signs of chondrocalcinosis. Diagnoses depicted are established at time of ascertainment and as published previously.[1]

Legend: age: age of patient at time of taking the radiograph; sex: M = Male, F = Female; OA: Radiographic osteoarthritis (Kellgren Lawrence grade 2 or higher in one or both joints; - = No radiographs available / no joint complaints; N: normal joint bilateral (Kellgren Lawrence grade 0 or 1); H: Heberden nodes; B: Bouchard nodes; C: Radiographic signs of chondrocalcinosis; ? = possible; DIP: distal interphalangeal joints; PIP: proximal interphalangeal joints; MCP: meta-carpo-phalangeal joints; CMCI: first-carpo-metacarpal joints; U/L: upper/lower segment ratio; P: Preclinical signs of osteoarthritis (bony enlargements / joint deformity / limited range of movement); BMI = body mass index (kg/m²).

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<th>Patient</th>
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<th>age</th>
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<th>PIP</th>
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<th>CMCI</th>
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<th>Shoulder</th>
<th>Hip</th>
<th>Knee</th>
<th>Feet</th>
<th>Ankle</th>
<th>CWK</th>
<th>LWK</th>
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OPG mutation directly causal to osteoarthritis

Supplementary Table 2. Variant prioritization scheme after exome sequencing.

<table>
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<th>Total number of variants identified</th>
<th>Subject 1</th>
<th>Subject 4</th>
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<tr>
<td>exonic (including 5'-utr and 3'-utr) and canonical splice sites</td>
<td>57018</td>
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<tr>
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<td>636</td>
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<tr>
<td>shared among distant family members</td>
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</table>

Supplementary Table 3. Genotyping of the detected variants in FOA family members.

*variant was not detected by genotyping. †DNA not available. ‡Total number of individuals showing linkage between phenotype and genotype (subjects with diagnostic uncertainty are not taken into account). SS = splice site.

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<th>DIS3</th>
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<td>I223T</td>
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<td>V209F</td>
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<td>*==&gt;L</td>
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<tr>
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<td>5/12</td>
<td>7/12</td>
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6
**Supplementary Table 4. Two point LOD scores FOA family a.** *LOD scores calculated with the original family structure and inheritance pattern[2]*
†LOD scores calculated with c.1205A=>T using the updated family structure.

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**Supplementary Table 5: Expression analyses of TNFRSF11B and TNFSF11 in articular cartilage.** Legend: Expr: relative gene expression, assessed by exploring a microarray mRNA expression dataset generated on Illumina HT-12 v3 chips in matched osteoarthritic and preserved cartilage samples of 33 patients that underwent joint replacement due to end stage osteoarthritis (expression levels are expressed as the light intensity values as provided by IlluminaTM. The average level of expression for all probes ranged from 6.58-14.91 with mean 7.4 and median 7.1 with a strong right tailing); Expr QT: Quartile of the expression level where 1 = lowest level of expression and 4 = highest level of expression; FC: fold change between osteoarthritic and preserved cartilage samples (FC > 1 indicated higher expression in osteoarthritic as compared to preserved cartilage).

TNYFRSF11B: tumor necrosis factor receptor superfamily member 11b encoding osteoprotegerin (OPG); TNFSF11: tumor necrosis factor (ligand) superfamily, member 11 encoding the nuclear factor-κB ligand (RANKL).

<table>
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<tr>
<th>Gene</th>
<th>Expr</th>
<th>Expr QT</th>
<th>FC</th>
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<tbody>
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<td>1.9 x 10^-8</td>
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<td>1.2</td>
<td>3.1 x 10^-6</td>
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</table>
Supplementary Figure 1. Schematic overview of the exome sequencing and follow-up analyses.

**Discovery**
Exome sequencing
- distant family members with early onset OA and chondrocalcinosis (N=2)
- 5 shared mutations

**Validation**
Genotyping
- OA patients (N=1467)
- Controls (N=744)
- Not detected

**Characterization**
Characterization of nt
- in vitro osteoclastogenesis & bone resorption
- Gain of function

**Causality**
Genotyping and Linkage analysis
- FOA family (N=16)
- OPG

**Generalizability**
Cartilage GeneExpr.
- Preserved vs OA (N=33)
- Up-regulation OA vs P (P-value=1.9x10^-4)

Supplementary Figure 2. Knee radiographs. **A** Right knee joint of FOA family member one at age 53 years with diagnosed chondrocalcinosis (not clearly visible on this photo) and marked osteoarthritis medially but without evidence of epiphyseal dysplasia.[1] **B** Knee joint of healthy control.
Supplementary Figure 3. Amino acid sequence of wild-type and mutant (X402L) TNFRSF11B or osteoprotegerin. An T>A base pair change at the stop codon in exon 5 (A1205T) results in a STOP to Leucine amino acid change (X402L) in the TNFRSF11B gene. This read through mutation leads to a 19 amino acid elongation of the mutant protein.

Supplementary Figure 4. Bone homeostasis depends on the local OPG/RANK ratio. The RANK ligand (RANKL) competitively binds to the OPG decoy and RANK receptor. Binding of RANKL to the RANK receptor at the osteoclast precursor cell acts as an osteoclast differentiation and activation signal.
Supplementary Figure 5. Recombinant human osteoprotegerin (OPG). To confirm integrity of the recombinant proteins, Western blotting was performed using 10 μl of the concentrated tissue culture supernatant of HEK293T cells 3 days after transfection with either empty vector (sup), wild-type (wt OPG) or mutant OPG (mt OPG), as indicated above the lanes.