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MARCOS VINÍCIUS DORNELAS DE MORAES

ASPECTOS GENÉTICOS E TERAPÊUTICOS DA OSTEOGÊNESE IMPERFEITA

VITÓRIA

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia ponto focal Universidade Federal do Espírito Santo, como requisito parcial para obtenção do título de Doutor em Biotecnologia.

Orientador: Profª. Drª. Flavia de Paula

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Dedico esta tese à minha família, que em nenhum momento mediu esforços para a realização dos meus sonhos, que me guiou pelos caminhos corretos, me ensinou a fazer as melhores escolhas, me mostrou que a honestidade e o respeito são essenciais à vida, e que devemos sempre lutar pelo que queremos. Com paixão e determinação, não há sonho impossível!

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ESTRUTURA DA TESE

Esta tese é apresentada em formato de artigos científicos.
As listas de figuras, quadros e siglas contêm as ilustrações, quadros e siglas apresentadas na introdução e na revisão bibliográfica deste trabalho.

RESUMO

A osteogênese imperfeita (OI) é uma desordem hereditária geneticamente heterogênea com incidência de 1 em cada 10-20.000 indivíduos nascidos. Caracteriza-se por deformidades no tecido conjuntivo e pela fragilidade óssea, o que torna o indivíduo com OI mais suscetível a fraturas, em razão de traumas mínimos ou impactos não traumáticos. A maioria dos casos a doença é herdada de forma autossômico dominante (AD) devido a uma mutação em heterozigose nos genes *COL1A1* ou *COL1A2*, que codificam, respectivamente, as cadeias de pró-colágeno α1(I) e α2(I) do colágeno tipo I, uma das proteínas estruturais mais importantes dos ossos, pele e tendões. Contudo, o número de relatos decorrentes de mutações autossômicas recessivas (AR) em novos genes é crescente, e já foram descritas mutações em 14 genes diretamente relacionados com a expressão clínica da OI, incluindo os genes *CRTAP, P3H1, PPIB, SERPINF1, SERPINH1*, *FKBP10, SP7 e WNT1*, foco deste trabalho. O uso de bifosfonatos por meio da administração intravenosa tem sido o tratamento padrão em OI. Os principais benefícios são a redução do número de fraturas, aumento de massa óssea e redução da dor crônica, o que contribui para o controle da progressão da doença uma melhora significativa na qualidade de vida dos pacientes.

Palavras-chave: Colágeno tipo I. Fragilidade óssea. Alterações genéticas. Correlação genótipo: fenótipo. Bifosfonatos.

ABSTRACT

Osteogenesis imperfecta (OI) is a genetically heterogeneous hereditary disorder with an incidence of 1 in every 10-20,000 individuals born. It is characterized by deformities in connective tissue and bone fragility, which makes the individual with OI more susceptible to fractures, due to minimal trauma or non-traumatic impacts. In most cases the disease is inherited in an autosomal dominant (AD) form due to a mutation in heterozygosity in the COL1A1 or COL1A2 genes, which encode the collagen α1(I) and α2(I) chains of the collagen type I, one of the most important structural proteins of bones, skin and tendons. However, the number of reports of autosomal recessive mutations (RA) in new genes is increasing, and mutations in 14 genes directly related to the clinical expression of OI, including *CRTAP, P3H1, PPIB, SERPINF1, SERPINH1, FKBP10, SP7* and *WNT1*, focus of this work. The use of bisphosphonates by intravenous administration has been the standard treatment in OI. The main benefits are the reduction of the number of fractures, increase of bone mass and reduction of chronic pain, which contributes to the control of the progression of the disease a significant improvement in the patients' quality of life.

Key words: Type I collagen. Bone fragility. Genetic alterations. Genotype correlation: phenotype. Bisphosphonates.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE SIGLAS

A	Adenina
AD	Autossômico Dominante
Ala	Alanina
AR	Autossômico Recessivo
Arg	Arginina
Asp	Ácido Aspártico
BMD	Densidade Mineral Óssea
cDNA	DNA complementar
C	Citosina
COL1A1	Gene que codifica a cadeia α1 do colágeno tipo I
COL1A2	Gene que codifica a cadeia α2 do colágeno tipo I
CRTAP	Gene que codifica a *cartilage-associated protein*
Cys	Cisteína
DMSO	Dimetilsufóxido
DNA	Ácido Desoxirribonucléico
dNTP	Deoxinucleotídeos Trifosfatados
FKBP10	Gene que codifica a *FK506-binding protein 65*
G	Guanina
GH	Hormônio do Crescimento
Glu	Glutamina

Gly	Glicina
Leu	Leucina
mRNA	RNA mensageiro
OI	Osteogênese Imperfeita
P3H1	Gene que codifica a *prolyl 3-hydroxylase 1*
PCR	Reação em Cadeia da Polimerase
PPIB	Gene que codifica a *ciclofilina B*
RNA	Ácido Ribonucléico
RNAi	RNA de interferência
Ser	Serina
SERPINF1	Gene que codifica um inibidor de serino-proteases
SERPINH1	Gene que codifica a *heat shock protein 47*
siRNAs	Pequenos RNAs de Interferência
SP7	Gene de um fator de transcrição específico de osteoblasto
SSCP	Polimorfismo Conformacional de Fita Simples
ssDNA	DNA Fita Simples
T	Timina
Val	Valina
WNT1	Gene que codifica a proteína Sinalizadora WNT

SUMÁRIO

1 INTRODUÇÃO

As doenças Mendelianas têm sido cada vez mais o foco das atenções quando o assunto envolve aplicações de técnicas biotecnológicas em Saúde. Devido ao crescente aumento na expectativa de vida e a presença dessas doenças, novos métodos para o diagnóstico e tratamento adequados vêm sendo desenvolvidos. A Biotecnologia tem permitido diagnosticar diferentes doenças com base no emprego de técnicas de Biologia Molecular e Bioinformática.

A Osteogênese Imperfeita (OI) é uma doença genética heterogênea caracterizada pela fragilidade óssea e predisposição a fraturas ósseas. A OI afeta 1 em cada 15-20.000 indivíduos e é tradicionalmente classificada em quatro tipos, com base nos aspectos clínicos e radiológicos. O tipo I é a forma leve, o tipo II é a forma letal, o tipo III é a forma mais grave não letal e o tipo IV é a forma moderada da doença (SILLENCE, SENN E DANKS, 1979).

A OI com padrão de herança autossômico dominante advém, em geral, de mutações nos genes estruturais que codificam as cadeias de pró-colágeno do colágeno tipo I, *COL1A1* e *COL1A2*, uma das proteínas mais importantes de pele, ossos e tendões (BARSH; BYERS, 1981). No entanto, é crescente o número de genes reportados para as formas autossômico recessivas (AR) da OI. Já foram descritas mutações em 14 genes diretamente relacionados com a expressão clínica da doença, incluindo os genes *CRTAP, P3H1, PPIB, SERPINF1, SERPINH1, FKBP10, SP7* e *WNT1*, os 8 mais frequentemente mutados na forma recessiva doença (DALGLEISH, 1998).

O diagnóstico clínico é bastante difícil em razão da associação dos sinais da OI com outras patologias. O diagnóstico precoce e preciso reflete na elaboração de processos e aplicações biotecnológicas impactantes sobre a qualidade de vida dos afetados pela doença. Assim, o propósito do presente trabalho foi investigar as alterações genéticas causadoras da doença e avaliar os efeitos do tratamento com pamidronato dissódico em pacientes com OI.

2 REVISÃO BIBLIOGRÁFICA

2.1 Histórico, Caracterização e Classificação Clínica da OI

No século XVII, a *“osteogenesis imperfecta”* (OI) era inicialmente denominada de osteomalácia congênita. O termo atual só foi adotado no final do século XIX e início do século XX (ROUGHLEY; RAUCH; GLORIEUX, 2003).

A manifestação da OI foi observada há mais de 3.000 anos atrás, por meio de um crânio parcialmente reconstituído, vestígios de uma múmia egípcia datada do ano 1.000 A.C., cujas peculiaridades nele observadas indicaram pertencer a uma criança afetada por OI (GRAY, 1969). Investigações posteriores detalharam a presença de alterações esqueléticas, como ossos finos e frágeis, além de dentinogênese imperfeita (LOWENSTEIN, 2009).

Ekman (apud WEIL, 1981) em 1788 elaborou uma tese sobre osteomalácia congênita descrevendo três gerações de uma família que apresentava fragilidade óssea hereditária e deformidades graves, mas com ausência de certas características como esclerótica azulada e surdez.

Axmann (apud SILLENCE; SENN; DANKS, 1979) em 1831 descreveu a doença associando a fragilidade óssea e a esclerótica azulada.

Lobstein (apud WEIL, 1981) em 1833 descreveu três casos de uma forma grave de fragilidade óssea em adultos de uma mesma família, denominando-a de *"osteopsatirose idiopática"* (KIM; COE; CHIN, 1970).

A forma congênita da doença posteriormente por Vrolik (apud WEIL, 1981), em 1849, que identificou pela primeira vez a doença em um recém-nascido que apresentou múltiplas fraturas ao nascimento, ossos *wormianos*, evoluindo à óbito perinatal. Foi quando surgiu o termo *“osteogenesis imperfecta”,* doença genética caracterizada pela fragilidade óssea (KIM; COE; CHIN, 1970).

Schmidt (apud WEIL, 1981) em 1897 observou que os fenótipos expressos tanto em adultos quanto em crianças na OI e na *"osteopsatirose idiopática"* tratavam-se de uma mesma enfermidade.

Spurway (1897) destacou a presença da esclerótica azulada em pacientes com fragilidade óssea. Eddowes (1900) propôs a mesma associação, o que auxiliou a determinar a coloração azulada em escleróticas como um dos sinais da OI.

Looser (1906), após analisar as semelhanças histológicas ósseas da OI e da osteopsatirose idiopática, classificou a OI em duas formas: (a) congênita ou doença de Vrolik, caracterizada pela presença de múltiplas fraturas ao nascimento, e (b) tardia ou doença de Eckamnn-Lobstein, onde as fraturas ocorreriam após o período perinatal.

Adair-Dighton (apud WEIL, 1981) descreveu pela primeira vez a perda auditiva associada à fragilidade óssea e esclerótica azulada em 1912. Posteriormente, Preiswerk descreveu as alterações dentárias peculiares da OI (MEDINA; LICÉAGA, 2010).

Van der Hoeve e de Kleijn (1918) associaram a surdez e otosclerose ao descrever uma família com três membros afetados em quatro gerações com as três características clássicas da OI. A conclusão de que os três sintomas principais (a) fragilidade óssea, (b) esclerótica azulada e (c) surdez tratavam-se da tríade clássica da OI foi de difícil resolução em razão de diferentes relatos (MARINI, 1988; MEDINA; LICÉAGA, 2010; PEDERSEN; ELBROND, 1979). Até que em 1928, Bell (apud RODGER, 1936) revisou mais de 300 casos de OI e pôde constatar a presença da tríade clínica em 44% dos mesmos.

A osteogênese imperfeita (OI) é uma desordem hereditária geneticamente heterogênea que ocorre igualmente em todos os grupos étnico-raciais. Com frequência de ocorrência variável de 1 em cada 10.000 a 20.000 indivíduos nascidos, a OI é caracterizada por deformidades no tecido conjuntivo e pela fragilidade óssea, o que torna o indivíduo com OI mais suscetível a fraturas, em razão de traumas mínimos ou impactos não traumáticos (BODIAN et al., 2009; ALANAY et al., 2010).

O diagnóstico clínico da OI é determinado pela observância de sinais e sintomas peculiares, tais como baixa estatura, esclerótica azulada, deformidades ósseas, fraturas ósseas, hipermobilidade articular, macrocefalia, dentinogênese imperfeita e surdez progressiva, entre outros (BARNES et al., 2010; BODIAN et al., 2009; BONADIO; RAMIREZ; BARR, 1990; CABRAL et al., 2003; CABRAL et al., 2007; CHANG; LIN; HSU, 2007; HARTIKKA et al., 2004; HUBER, 2007; KATAOKA et al., 2007; MARINI et al., 2007a; RAUCH; GLORIEUX, 2004; SANTILI et al., 2005).

Com uma expressão fenotípica bastante variável, a OI compreende desde formas mais graves, com letalidade perinatal e múltiplas fraturas intrauterinas, até casos em que o diagnóstico clínico da doença passa desapercebido devido à ausência de fraturas ou deformidades ósseas e mobilidade normal. Acredita-se que casos mais leves da doença sejam subestimados diante da dificuldade de se realizar um diagnóstico preciso (GLORIEUX, 2008; RAUCH; GLORIEUX, 2004; SILLENCE; SENN; DANKS, 1979).

A OI advém frequentemente de mutações *de novo* ou por mosaicismo gonadal parental, provenientes de pais saudáveis e sem histórico clínico de doenças significantes (MAASALU et al., 2015; FREDERIKSEN et al., 2016). No entanto, na maioria dos casos a doença é herdada de forma autossômica dominante (AD) devido a uma mutação em heterozigose nos genes *COL1A1* ou *COL1A2*, que codificam, respectivamente, as cadeias de pró-colágeno α1(I) e α2(I) do colágeno tipo I, uma das proteínas estruturais mais importantes dos ossos, pele e tendões (BARSH; BYERS, 1981). Em adição, já foram observadas mutações autossômicas dominantes no gene *IFITM5*, responsável pela codificação de uma proteína transmembrana osteoblasto-específica relacionada à mineralização óssea (LAZARUS et al., 2014).

O número de relatos decorrentes de mutações autossômicas recessivas (AR) em novos genes é crescente, sendo descritas mutações em 14 genes diretamente relacionados com a expressão clínica da OI (DALGLEISH, 1998), incluindo os genes *CRTAP, P3H1, PPIB, SERPINF1, SERPINH1*, *FKBP10, SP7 e WNT1*, foco deste trabalho.

O uso de bifosfonatos, tais como o pamidronato dissódico, por meio da administração intravenosa em adultos e particularmente crianças com Osteogenesis Imperfecta tem sido o tratamento padrão em OI. Os principais benefícios são a redução do número de fraturas, aumento de massa óssea e redução da dor crônica, o que contribui para o controle da progressão da doença uma melhora significativa na qualidade de vida dos pacientes (BRIGGS et al. 2015).

Notoriamente, a OI trata-se não apenas de uma entidade única, mas de uma família de anormalidades semelhantes que compartilham uma tendência à fragilidade óssea e ocorrência de fraturas. Em 1949, na primeira tentativa de classificação da doença, Seedorf (apud SILLENCE; SENN; DANKS, 1979) subclassificou a OI em Tipo I (ou congênita, conforme determinado por Looser); Tipo II (tardia grave), na qual a primeira fratura ocorre no nascimento ou no primeiro ano de vida, com deformidade dos ossos longos e coluna; Tipo III (tardia leve), com deformidades leves ou ausentes e fraturas após o primeiro ano de vida. No entanto, dada a heterogeneidade clínica da doença, Sillence e colaboradores apresentaram em 1979 uma classificação em quatro tipos clínicos da OI (I-IV), com base em critérios clínicos, radiológicos e genéticos (CHANG et al., 2010).

A OI tipo I é a forma leve, na qual a heterogeneidade intrafamiliar e interfamiliar é significativa. Os indivíduos afetados apresentam esclerótica azulada, estatura normal ou levemente baixa, surdez precoce em cerca de 50% dos pacientes, fragilidade óssea variável, um risco aumentado de fraturas em decorrência de traumas leves que habitualmente ocorrem com o início da deambulação e/ou deformidades ósseas mínimas em radiologia, como uma leve osteopenia, ossos com cortical fina e crânio com ossos *wormianos* em mosaico. A OI tipo I ainda pode ser subclassificada com base na ausência (IA) ou presença (IB) de dentinogênese imperfeita (CHEUNG; GLORIEUX, 2008; GAJKO-GALICKA, 2002; HUBER, 2007; KANEKO et al., 2011; LEVIN; SALINAS; JORGENSON, 1978; PRIMORAC et al., 2001; RAUCH et al., 2003; ROUGHLEY; RAUCH; GLORIEUX, 2003).

A OI tipo II foi subdividida em três subtipos distintos por Sillence e colaboradores (1979), com base nos achados clínicos e radiológicos. O subtipo IIA representa a forma mais grave, associada à letalidade no período perinatal, com múltiplas fraturas intrauterinas e deformidades ósseas. Caracteriza-se por esclerótica

azulada/acinzentada, proptose, deficiência na ossificação craniana, estatura e peso baixos. Os achados radiológicos são ossos pouco mineralizados, largos e curtos, ossos *wormianos* em mosaico, platispondilia, fêmures em fita, múltiplas fraturas e costelas em rosário. A insuficiência respiratória é a principal causa de morte devido a fraturas múltiplas de costelas e insuficiência pulmonar. O subtipo IIC é uma forma grave de OI com face triangular, protusão ocular, hipertelorismo, deficiência na ossificação craniana, extremidades longas e relativamente encurvadas. As principais características radiológicas são escápulas com forma e ossificação irregulares, ísquios longos e angulados, ossos longos finos e encurvados, sendo a coluna praticamente normal. O subtipo IIB não pode ser diferenciado clínica ou radiologicamente do tipo III (BYERS et al. 1988; CHEUNG, GLORIEUX, 2008; GAJKO-GALICKA, 2002; KANEKO et al., 2011; YOUNG et al. 1987).

A OI tipo III (similar ao tipo IIB) é compatível com a sobrevida após o período neonatal e ao nascimento, observa-se pelas fraturas múltiplas e deformidades ósseas resultantes de fraturas intrauterinas. Caracteriza-se por esclerótica azulada, *caput* membranáceo, baixa estatura (varia de 90 a 120cm), escoliose e, ocasionalmente, dentinogênese imperfeita. Frequentemente apresenta perda auditiva progressiva. Os achados radiológicos são osteopenia, costelas finas com fraturas descontínuas, platispondilia, ossos *wormianos* em mosaico, ossos tubulares encurtados com metáfises alargadas. Estes indivíduos fraturam com maior frequência do que em qualquer outro tipo de OI e apresenta expectativa de vida relativamente curta. A deformidade óssea progressiva de ossos longos e de coluna está associada com fraturas de repetição e com a própria heterogeneidade genética da doença, o que pode requerer múltiplos procedimentos de correção ortopédica (haste intramedular) e locomoção com auxílio de cadeira de rodas. O óbito na infância pode ocorrer por problemas respiratórios em virtude de comprometimento torácico ou por traumas, como fratura craniana (BYERS et al. 1988; CHEUNG & GLORIEUX, 2008; CHRISTIANSEN et al. 2010; GAJKO-GALICKA, 2002; HUBER, 2007; KUIVANIEMI; TROMP; PROCKOP, 1997; PRIMORAC et al., 2001; ROUGHLEY; RAUCH; GLORIEUX, 2003).

A OI tipo IV representa o grupo de maior variabilidade fenotípica (intrafamilial e interfamilial) da classificação de Sillence, o que sugere forte heterogeneidade

genética, uma vez que engloba todos os indivíduos que não se enquadram nos demais tipos. Com similaridades entre os tipos I e III, o fenótipo pode variar de leve (com mobilidade normal) a grave (dependente de cadeira de rodas), com observação de fraturas e deformidades ósseas ao nascimento, além da expectativa de vida menor. Caracteriza-se pela presença de esclerótica normal ou levemente acinzentada, deformidades ósseas de leve a grave e perda auditiva (é menos comum do que no tipo I). Frequentemente, a baixa estatura e dentinogênese imperfeita (ausente no tipo IVA e presente no tipo IVB) podem ser observadas. Não há retardo no crescimento intrauterino, mas o crescimento pós-natal é bem reduzido e segue baixos percentis. Outras manifestações incluem hipermobilidade articular e escoliose, variando de leve a severa. As fraturas costumam quiescer entre os 20 e 40 anos de idade, sendo mais frequentes da infância à puberdade e em idades mais avançadas (BYERS et al. 1988; CHEUNG; GLORIEUX, 2008; GAJKO-GALICKA, 2002; HANSCOM; BLOOM, 1988; HUBER, 2007; LAPUNZINA et al. 2010; PRIMORAC et al., 2001; RAUCH; GLORIEUX, 2004; ROUGHLEY; RAUCH; GLORIEUX, 2003).

Os tipos V e VI, eram previamente classificadas como tipo IV. O tipo V é caracterizado pela fragilidade óssea e osteopenia de moderada a severa e ausência de esclerótica azulada ou dentinogênese imperfeita. Ocorrem três peculiaridades distintas: (a) o frequente desenvolvimento de calo hipertrófico nos sítios de fratura, após cura ou cirurgia corretiva, mimetizando osteosarcoma; (b) a calcificação de membranas interósseas entre os ossos do antebraço, o que pode limitar movimentos de pronação e a supinação e, secundariamente, provocar o deslocamento da cabeça do rádio e, (c) a presença em radiologias de bandas metafásicas radiopacas adjacentes às fises (anéis de crescimento). A análise histomorfométrica de biópsia de crista ilíaca revela lamelação óssea irregular do tipo *mesh-like*, claramente distinta do que ocorre em OI tipos I e IV (CHEUNG; GLORIEUX, 2008; GLORIEUX, 2008; HUBER, 2007; KANEKO et al., 2011; PRIMORAC et al., 2001; RAUCH; GLORIEUX, 2004; ROUGHLEY; RAUCH; GLORIEUX, 2003).

A OI tipo VI resulta em fraturas mais frequentes do que aqueles com OI tipo IV e todos apresentam fraturas de compressão vertebral. Também apresentam graus de deformidade esquelética e fragilidade óssea de moderados a severos, com ausência

de dentinogênese imperfeita, esclerótica de coloração normal ou discretamente azul, estatura moderada e escoliose. A análise histomorfométrica de biópsia de crista ilíaca revela lamelação óssea irregular com um padrão do tipo *fish-scale*, além da presença excessiva de osteóides nas superfícies ósseas (ALANAY et al., 2010; CHEUNG; GLORIEUX, 2008; GLORIEUX, 2008; HUBER, 2007; KANEKO et al., 2011; PRIMORAC et al., 2001; RAUCH; GLORIEUX, 2004; ROUGHLEY; RAUCH; GLORIEUX, 2003).

Em razão da grande complexidade genética e variabilidade fenotípica advinda dos diferentes loci descritos ultimamente, a classificação com base nos tipos de Sillence tornou-se insuficiente para a prática clínica. Assim, a classificação tradicional de Sillence vem sendo continuamente revista e expandida no intuito de incluir os dados clínicos, histológicos, radiológicos e genéticos recém observados. Diferentes revisões de nomenclatura culminaram em 15 tipos de OI (OI tipo I-XV), além de tipos clínicos sem classificação específica.

Tais propostas têm se tornado cada vez mais confusas no âmbito clínico, o que resultou numa classificação na qual os tipos clínicos não são mutuamente exclusivos. OI tipos I-IV foram primariamente definidos com base em características clínicas e radiológicas, enquanto que os novos tipos (com exceção do tipo V) foram definidos em razão de novos loci descobertos, mas com características clínicas e radiológicas comparáveis aos tipos II-IV. A tabela 1 reúne os tipos clínicos com base em critérios genéticos, com base em referências que trataram extensivamente do assunto bem como publicações recentes (FORLINO et al., 2011; MARINI & BLISSETT, 2013; VALADARES et al., 2014; MARINI; REICH; SMITH, 2014; MENDOZA-LONDONO et al., 2015; KANETO et al., 2016).

A considerável variabilidade fenotípica intrafamiliar e interfamiliar torna a classificação clínica um processo arduamente difícil e complexo, o que requer observações mais aprofundadas acerca das diferentes peculiaridades clínicas apresentadas pelos indivíduos afetados. Este fato foi determinante para a criação de critérios internacionais comuns para classificar a gravidade da OI entre indivíduos afetados, bem como as possibilidades em tratamento, sejam por métodos cirúrgicos, farmacológicos ou conservadores.

Tabela 1 – Classificação da Osteogênese Imperfeita

TIPO DE OI		HERANÇA	GENE	FENÓTIPO
Tipos Clássicos (SILLENCE; SENN; DANKS, 1979)	I	AD	*COL1A1* *(alelo nulo)*	Leve; Sem Deformidade
	II	AD	*COL1A1/2*	Letal Perinatal
	III	AD	*COL1A1/2*	Deformidade Progressiva
	IV	AD	*COL1A1/2*	Deformidade Moderada
Defeitos de Proteína de Matrix Extracelular; Sem Mutações em *COL1*	IV	AR	*SPARC*	Moderado
Defeitos de Mineralização	V	AR	*IFITM5*	Moderado; Histológico Distinto
	VI	AR	*SERPINF1*	Moderado a Severo; Histológico Distinto
Defeitos de 3-Hidroxilação	VII	AR	*CRTAP*	Severo a Letal
	VIII	AR	*P3H1*	Severo a Letal
	IX	AR	*PPIB*	Moderado a Letal
Defeitos de Chaperonas	X	AR	*SERPINH1*	Severo
	XI	AR	*FKBP10*	Deformidade Progressiva (Síndrome de Bruck)
Defeitos de Maturação de Osteoblasto	XII	AR	*SP7*	Moderado
Defeitos de Clivagem C-propeptídica	XIII	AR	*BMP1*	Severo, Alta Densidade Óssea
Defeitos de Canal Catiônico	XIV	AR	*TMEM38B*	Moderado a Severo
Defeitos da Proteína Sinalizadora *WNT*	XV	AR	*WNT1*	Variável
	XV	AD	*WNT1*	Osteoporose Precoce
Defeitos de Transcrição em *COL1A1*	-	AR	*CREB3L1*	Severo a Letal
Defeito de Osteócitos	-	XL	*PLS3*	Leve; Osteoporose e Fraturas sem Hipermineralização de Matriz Óssea
Defeitos de Hidroxilação Telopeptídica do Colágeno	-	AR	*PLOD2*	Deformidade Progressiva (Síndrome de Bruck)

AD, autossômico dominante; AR, autossômico recessivo; XL, herança ligada ao X (FORLINO et al., 2011; MARINI & BLISSETT, 2013; VALADARES et al., 2014; MARINI et al., 2014; MENDOZA-LONDONO et al., 2015; KANETO et al., 2016).

A escala de classificação de gravidade da doença proposta recentemente está em fase de validação em diferentes centros especializados e contempla dados clínicos, históricos, frequência de fraturas, densitometria óssea, e nível de mobilidade, além de um guia geral para dados clínicos pré-natais e dados de ultrassonografia (VAN DIJK; SILLENCE, 2014).

2.2 Aspectos Bioquímicos, Genéticos e Moleculares da OI

A manifestação clínica da OI, representada principalmente pela fragilidade do tecido ósseo, é decorrente do resultado das mutações em genes associados ao colágeno tipo I sobre as estruturas ósseas, uma vez que o colágeno tipo I é a principal proteína constituinte dos ossos. Os ossos são constituídos por células ósseas (osteoblasto, osteócitos e osteoclastos), matriz orgânica (colágeno e proteoglicanas) e minerais (fosfato de cálcio depositado sob a forma de hidroxiapatita), sendo um reservatório de minerais essenciais com aproximadamente 99% do cálcio, 85% do fósforo e até 60% do sódio e potássio corporais (HUNTER; GOLDBERG, 1993; HUBER, 2007; BAHT; HUNTER; GOLDBERG, 2008).

Os osteoblastos, derivados de células-tronco mesenquimais presentes na medula óssea e superfícies ósseas onde a matriz está sendo formada, produzem e secretam a maior parte da matriz orgânica e regula a sua mineralização. Sintetizam o colágeno tipo I, o mais abundante da matriz orgânica, assim como proteoglicanas, glicoproteínas entre outras proteínas. Regulam a diferenciação e a atividade absortiva osteoclástica. Podem entrar em apoptose após um período de atividade secretória, como também podem se incorporar à matriz óssea através da diferenciação em osteócitos (MACKIE, 2003).

Os osteócitos, presentes em maior quantidade no tecido ósseo maduro, localizam-se em lacunas internas e se comunicam por uma rede de conexões constituída por processos intracanaliculares que permitem o trânsito de metabólitos, íons e sinalizadoras intracelulares. Participam dos processos de manutenção, formação,

viabilidade e reabsorção da matriz e dos minerais pela osteólise osteocítica, mantendo constantes os níveis de cálcio extracelulares, além de imprimirem sinais bioquímicos que regulam o *turnover* ósseo em resposta a forças mecânicas (AARDEN; NIJWEIDE; BURGER, 1994; OCARINO et al., 2006).

Os osteoclastos são células multinucleadas derivados da fusão de precursores das células mononucleares (monócitos e macrófagos) hematopoiéticas com diferenciação dependente de estímulos liberados por osteoblastos, culminando com o início do remodelamento ósseo. Localizam-se na superfície das trabéculas e dos canais haversianos e no periósteo, alojados nas lacunas de Howship. Os osteoclastos têm a capacidade de erodir tanto a matriz orgânica quanto a matriz mineral por osteoclasia (ADEBISI, 2009; OCARINO; SERAKIDES, 2006).

Das proteínas não colágenas dos ossos que compõem cerca de 10% da matriz orgânica, as proteoglicanas de matriz e as glicoproteínas são as mais abundantes e desempenham um papel importante na mineralização e na reabsorção óssea. A osteopontina e sialoproteína óssea são proteínas necessárias para o início da mineralização óssea e atuam como nucleadoras de cristais. Já a osteocalcina e a osteonectina estão presentes na matriz totalmente mineralizada e podem estar associadas com o controle do processo de formação de cristais (tamanho e forma) e velocidade de formação. Durante a reabsorção óssea, a osteocalcina atua como agente quimiotactante de osteoclastos enquanto que a osteopontina e a sialoproteína óssea parecem facilitar a associação dos osteoclastos à matriz óssea (ROACH, 1994).

Dentre os diferentes tipos de colágeno, o colágeno tipo I é a proteína extracelular mais abundante no osso, representando até 90-95% da matriz óssea orgânica. É também um dos principais constituintes dos tendões, ligamentos, pele, dentes e fáscias, entre outros (BARSH; BYERS, 1981; BECK et al., 2000; GAJKO-GALICKA, 2002; HUBER, 2007; POPE et al., 1985; PROCKOP; KIVIRIKKO, 1984, 1995).

2.2.1 Defeitos na Síntese ou Estrutura do Colágeno Tipo I (COL1A1, COL1A2)

A maioria dos pacientes (90%) com OI tipos I a IV tem mutações dominantes em um dos genes que codificam as cadeias de pró-colágeno α1(I) e α2(I), subunidades estruturais da tripla hélice do colágeno tipo I, *COL1A1* (MIM# 120150) no cromossomo 17q23.3-q22 e *COL1A2* (MIM# 120160) no cromossomo 7q21.3–q22, respectivamente. Ambos os genes *COL1A1* e *COL1A2* possuem 52 exons e geram mRNAs de tamanhos parecidos (DE WET et al., 1987; GAJKO-GALICKA, 2002; HUBER, 2007; KUIVANIEMI et al., 1988; MARINI et al., 2007a; PACE et al., 2001; PROCKOP; KIVIRIKKO, 1995; RAMSHAW, 1998; RAUCH; GLORIEUX, 2004; TROMP et al., 1988; WITECKA et al., 2008).

O pró-colágeno tipo I, precursor do colágeno tipo I, é constituído por um heterotrímero de duas cadeias α1(I) e uma cadeia α2(I), e consiste de uma estrutura super enovelada em tripla hélice construída a partir de três cadeias extensas de poliprolina tipo II. As cadeias consistem de um peptídeo sinal, extremidades pró-peptídicas não colágenas N-terminal e C-terminal, telopeptídeos N-terminal e C-terminal, e de um domínio colágeno longo e helicoidal, conforme indicado na ilustração 1 (HUBER, 2007; MARINI et al., 2007a; PACE et al., 2001; PROCKOP; KIVIRIKKO, 1995; RAMSHAW, 1998; WITECKA et al., 2008).

Os 43 exons que codificam o domínio em tripla hélice do colágeno, parte funcional da molécula que provê força de tensão e serve como molde para a deposição de minerais nos ossos, são compostos de 338 *triplets* (trincas) de aminoácidos Gly-Xaa-Yaa, gerando um padrão de sequência repetitiva do tipo $(Gly\text{-}X\text{-}Y)_n$. O *triplet* mais frequente é *Gly-Pro-Hyp*, que também contribui para a máxima estabilidade da tripla hélice. Em adição ao alto conteúdo de aminoácidos prolina e hidroxiprolina, a tripla hélice também é estabilizada pelo íntimo dobramento das três cadeias graças à presença da glicina a cada terceiro resíduo, por pontes de hidrogênio entre as cadeias e por uma extensiva rede de hidratação. A glicina (Gly), que possui a cadeia lateral composta por um único hidrogênio, é essencial a cada terceira posição da cadeia (próximo ao centro da hélice) por ser o único resíduo pequeno o suficiente para permitir a formação de uma íntima e apertada estrutura helicoidalmente torcida

sem que haja qualquer distorção (BAUM; BRODSKY, 1997; BECK et al., 2000; BUEVICH et al., 2004; BYERS, 2000; HUBER, 2007; MARAKAREEVA et al., 2008; MARINI et al., 2007a, 2007b; PACE et al, 2001; PROCKOP; KIVIRIKKO, 1995; RAMSHAW, 1998; WITECKA et al., 2008).

In vivo, as moléculas de colágeno Tipo I são sintetizadas sob a forma de pró-colágeno. Os mRNAs maduros precursores destas moléculas são traduzidos junto à membrana dos polissomos se associam no lúmen do retículo endoplasmático rugoso. As moléculas de pró-colágenos maduros são transportadas para o Golgi e empacotadas em vesículas de transporte, onde ocorre a agregação lateral – algumas prolinas e lisinas na posição Y são hidroxiladas, e alguns resíduos de lisina podem ser subsequentemente glicosilados por glicosiltransferases. A região C-terminal, responsável pelo processo de trimerização (seleção e o alinhamento cadeia-cadeia, montagem dos constituintes das cadeias e o registro apropriado do domínio helicoidal triplo), não apresenta obrigatoriedade pela sequência $(Gly\text{-}X\text{-}Y)_n$ e contêm diversos resíduos de cisteína e triptofano, ausentes no domínio funcional do colágeno, que podem formar pontes dissulfeto intracadeias. Estes resíduos são conservados e desempenham papéis cruciais nos estágios iniciais de montagem do pró-colágeno. Os demais participam de ligações covalentes entre cadeias do mesmo trímero.

A nucleação C-terminal através da sequência $(Gly\text{-}Pro\text{-}Hyp)_5$ e a subsequente formação da tripla hélice é propagada linearmente em direção a região N-terminal das cadeias, conduzida por um mecanismo em forma de zíper sob uma taxa limitada pela lenta isomerização *cis-trans* das pontes de aminoácidos (BAUM; BRODSKY, 1997; BECK et al., 2000; BUEVICH et al., 2004; BYERS, 2000; HUBER, 2007; MARAKAREEVA et al., 2008; MARINI et al., 2007a, 2007b; PACE et al, 2001).

Uma vez produzida e secretada para o espaço pericelular, ocorre a clivagem das extremidades globulares N-terminal e C-terminal para criar moléculas de colágeno funcionais, as quais se auto-associam em um padrão escalonado para formar as fibrilas de 300nm (BAUM; BRODSKY, 1997; BECK et al., 2000; BUEVICH et al., 2004; BYERS, 2000; HUBER, 2007; MARAKAREEVA et al., 2008; MARINI et al., 2007a, 2007b; PACE et al, 2001; PROCKOP; KIVIRIKKO, 1995; RAMSHAW, 1998; WITECKA et al., 2008).

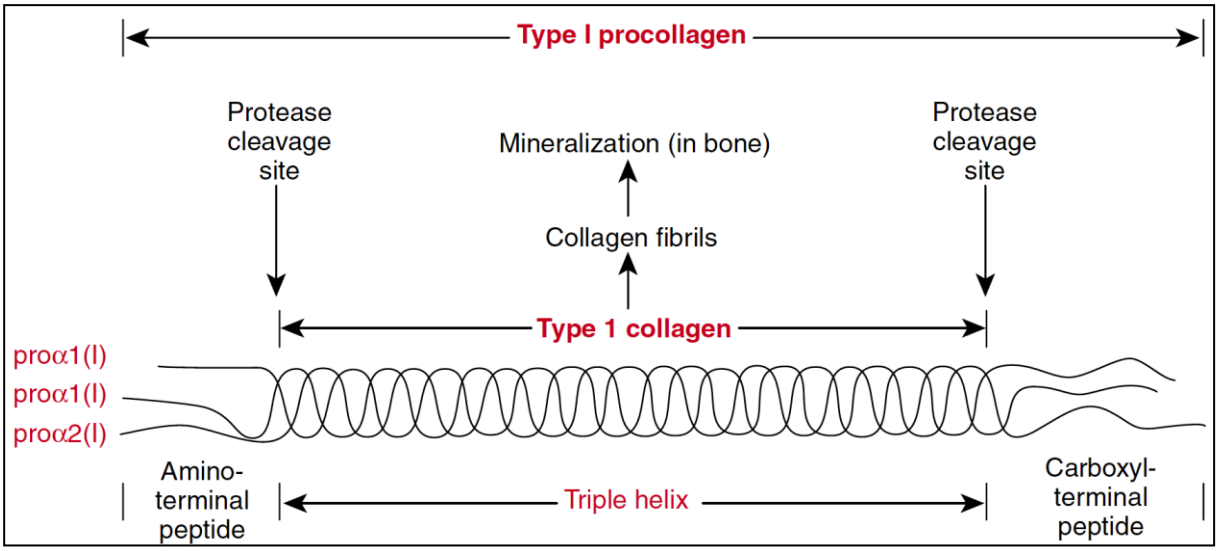


Ilustração 1 – Estrutura do pró-colágeno tipo I. O pró-colágeno tipo I é composto por duas cadeias α1(I) e uma α2(I). A interação entre cadeias de pró-colágeno α1(I) e α2(I) resulta em uma estrutura em tripla hélice que é secretado no espaço pericelular. Os domínios N e C-terminal são clivados extracelularmente para formar colágeno. As fibrilas de colágeno maduro agregam-se entre si e, nos ossos, são mineralizadas (WALTER BURNS SAUNDERS COMPANY, acesso em 27 out. 2017 - Modificado).

2.2.2 Defeitos no Complexo de 3-Hidroxilação (CRTAP, P3H1, PPIB)

O gene *CRTAP* (MIM# 605497), *cartilage associated protein*, está localizado no cromossomo 3p22.3, possui aproximadamente 6kb de extensão (BARNES et al., 2006; CASTAGNOLA et al., 1997; MARINI; CABRAL; BARNES, 2010; MORELLO et al., 2006; TONACHINI, L. et al. 1999). O gene *P3H1* (MIM# 610339), *prolyl 3-hydroxylase 1*, está localizado no cromossomo 1p34.1. Codifica uma enzima de 84kDa da família das prolil hidroxilases (JARNUM et al., 2004; KAUL et al., 2000; MARINI; CABRAL; BARNES, 2010; VRANKA; SAKAI; BACHINGER, 2004). O gene *PPIB* (MIM# 123841), *peptidyl-prolyl isomerase B (cyclophilin B)*, está localizado no cromossomo 15q21-q22. Codifica uma proteína de 21kDa de ligação de ciclosporinas (BARNES et al., 2010; PRICE et al., 1991; MARINI et. al., 2007b).

Juntos, esses três elementos formam um complexo molecular no retículo endoplasmático rugoso na razão de 1:1:1. A α1(I)Pro986 hidroxilação é uma de muitas modificações das cadeias de pró-colágeno que contribui para o correto

dobramento, estabilidade e secreção do pró-colageno. Prolil 4-hidroxilação é importante para a estabilidade térmica da tripla hélice colágena enquanto que a hidroxilação de lisinas e glicosilação de hidroxilisinas contribuem para uma estabilidade na interação extracellular entre moléculas.

Nas formas humanas de OI, mutações em homozigose ou heterozigose composta nos genes *CRTAP* e *P3H1* estão associados a modificações pós-traducionais excessivas em cadeias de colágeno tipo I sintetizadas por fibroblastos cultivados in vitro, o que causa retardo na mobilidade eletroforética da proteína (BALDRIDGE et al., 2008). Por outro lado, a deficiência do gene *PPIB* resulta em osteogênese moderada sem rizomelia, associada com a normal α1(I)Pro986 hidroxilação e modificações normais da tripla hélice colágena I (BARNES et al., 2010).

2.2.3 Defeitos na Mineralização (SERPINF1)

O gene *SERPINF1* (MIM# 172860), *serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived fator*, *PEDF), member 1*, está localizado no cromossomo 17p13.3. Codifica uma proteína inibidora de serino-proteases (BECKER et al., 2011; TOMBRAN-TINK et al., 1994). É uma proteína multifunctional envolvida na homeostase óssea e um dos maiores inibidores de angiogênese conhecido em humanos. Na ausência de PEDF secretado por osteoblastos, as células precursoras de osteoclastos podem se diferenciar em osteoclastos sem nenhuma limitação, o que leva a uma excessiva reabsorção óssea. Os fenótipos de OI observados em pacientes com mutações em *SERPINF1* são o resultado de mecanismos primariamente independentes de alterações da síntese ou processamento intracelular do colágeno tipo I (BECKER et al., 2011).

2.2.4 Defeitos no Dobramento do Colágeno Tipo I (SERPINH1, FKBP10)

O gene *SERPINH1* (MIM# 600943), *serpin peptidase inhibitor, clade H (heat shock protein 47, HSP47), member 1, (collagen binding protein 1)*, está localizado no cromossomo 11q13.5. Codifica uma glicoproteína que se liga ao colágeno tipo I (CHRISTIANSEN et al. 2010; IKEGAWA et al.,1995). O gene *FKBP10* (MIM# 607063), *FK506 binding protein 10,* 65 kDa, está localizado no cromossomo 17q21.2. Codifica uma proteína da família das PPIases (*FKBP-type peptidyl-prolyl cis/trans isomerase*) presentes no retículo endoplasmático e atua também como uma chaperona por participar do dobramento do colágeno tipo I (ALANAY et al., 2010; PATTERSON et al.; 2000).

Existe uma interação entre as proteínas HSP47, FKBP65 e o colágeno tipo I dentro do RE, que resulta em OI quando há uma disfunção. Mutações do tipo missense em homozigose em HSP47 produz OI moderadamente severa e estabelece uma relação intracelular entre HSP47 e FKBP65, sugerindo que elas trabalham em conjunto durante a biossíntese do pró-colágeno tipo I. Mutantes de HSP47 levam a uma diminuição da proteína FKBP65. Enquanto que a perda de FKBP65 não afeta os níveis de HSP47, mas provoca a sua deslocalização. HSP47 transita com o pró-colágeno tipo I do RE para o ERGIC (Compartimento intermediário RE-Golgi).

Mutantes de HSP47 podem contribuir para a falha no transporte do pró-colágeno tipo I para o Golgi, o que leva a formação de vesículas intracelulares. Uma falha nessa cooperação altera o tráfego do pró-colágeno tipo I e leva a sua acumulação nas vesículas do RE. A presença de pró-colágeno tipo I em vesículas anormais implica em moléculas não sendo normalmente processadas através do Golgi e podem ser recicladas ou removidas juntamente com a chaperonas defeituosas (CHRISTIANSEN et al., 2010; DURAN et al., 2015).

2.2.5 Defeitos na Maturação de Osteoblastos (SP7)

O gene *SP7* (MIM# 606633), *SP7 transcription factor*, está localizado no cromossomo 12q13.13. Codifica um fator de transcrição dedo de zinco *C2H2-type*, específico do tecido ósseo (GAO et al., 2004; LAPUNZINA et al. 2010, NAKASHIMA et al., 2002).

O gene *SP7/OSX* está especificamente expresso em osteoblastos corticais e trabeculares e a baixos níveis em condrócitos pré-hipertróficos da placa de crescimento ósseo. Esse padrão de expressão restrito é consistente com pacientes tendo esclerótica de coloração branca normal. Tem um papel essencial na regulação da diferenciação de pré-osteoblastos em osteoblastos, e é um importante regulador da diferenciação de osteoblastos e da formação óssea (LAPUNZINA et al., 2010).

2.2.6 Defeitos da Proteína Sinalizadora WNT (WNT1)

O gene *WNT1* (MIM# 164820), wingless-type MMTV integration site family, member 1, está localizado no cromossomo 12q13.12. É uma proteína de ligação *WNT* importante para a regulação de massa óssea em humanos. Alelos hipofuncionais de *WNT1* causam fenótipos de baixa densidade óssea. Enquanto que mutações herdadas de forma recessiva e que provocam uma redução na função de WNT1 levam à casos de OI congênita. O fato de que mutações em heterozigose OI predispõe à osteoporose precoce implica em um efeito dominante (KEUPP et al., 2013).

A via canônica de sinalização de WNT é essencial para o correto desenvolvimento esquelético e homeostase. Ela induz a diferenciação osteoblástica e formação óssea em progenitores osteoblásticos precoces e regula a osteoclastogênese osteoblasto dependente em osteócitos e osteoblastos maduros (LAINE et al., 2013).

Mutações bialélicas de perda de função em *WNT1* resultam em cenários recessivos que incluem fragilidade óssea com fenótipos moderadamente severos e progressivos, que não são facilmente distinguíveis da OI tipo III (PYOTT et al., 2013).

2.3 Correlação Genótipo: Fenótipo

Mutações nos genes estruturais do colágeno tipo I são a causa principal de OI tipos I a IV com padrão de recorrência autossômico dominante. A expressão clínica da OI, frequentemente, advém de mutações *de novo* ou por mosaicismo gonadal parental. Variações genéticas nos demais genes da OI foram detectadas em pacientes com as formas grave ou letal, assim como as demais formas de OI, com um padrão de recorrência autossômico recessivo (DALGLEISH, 1998).

Atualmente estão descritas junto ao *Database of Human Osteogenesis Imperfecta and Type III Collagen Mutations* 1932 variantes genéticas para o gene *COL1A1*, e mais de 1009 para o gene *COL1A2* (DALGLEISH, 1998; ZHANG et al., 2011).

Existem duas classes de mutações no colágeno tipo I que resultam em OI: aquelas que causam um defeito quantitativo, resultado da síntese parcial (50%) da quantidade normal de pró-colágeno tipo I, e aquelas que resultam na síntese de moléculas de colágeno com anormalidades estruturais (Ilustração 3). As mutações que afetam α1(I) costumam originar fenótipos clínicos mais graves daqueles observados em α2(I), uma vez que o trímero de colágeno tipo I consiste de duas cadeias α1(I), mas apenas de uma cadeia α2(I) (MARINI et al., 2007a; RAUCH et al., 2010).

As mutações que provocam um defeito quantitativo normalmente provocam a falha na síntese de produtos de um dos alelos *COL1A1* ou no alelo *COL1A2*, o que caracteriza um quadro de haploinsuficiência do gene em virtude de mutações dos

tipos *frameshift*, *nonsense*, e mutações em sítios de *splicing*, fato comumente observado em indivíduos com quadro clínico de OI tipo I (MARINI et al., 2007a).

Já mutações que provocam a síntese de moléculas estruturalmente anormais de colágeno tipo I, advindas de alterações na sequência do domínio da tripla hélice da proteína – substituições da glicina constitutiva do *triplet Gly-X-Y* – resultam em um amplo espectro fenotípico com quadros clínicos variando de letal (OI tipo II) a leve (OI tipo I) (MARINI et al., 2007a).

Em se tratando de mutações que interferem qualitativamente na estrutura do colágeno tipo I, os fenótipos clínicos dependem de parâmetros como (a) a natureza da alteração (substituições, duplicações, inserções, deleções, *frameshifts*, *nonsense*, entre outras; (b) o aminoácido que substitui a glicina; (c) a posição da mutação ao longo da tripla hélice, domínio estrutural funcional da molécula de colágeno tipo I, além de possíveis fatores externos (qualidade de vida, fatores ambientais, entre outros) (MARINI et al., 2007a; ZHANG et al., 2011).

A substituição do aminoácido glicina por qualquer outro ao longo das cadeias de pró-colágeno tipo I interfere no dobramento desta molécula, o que ocasiona uma mineralização óssea precária e, consequentemente, a expressão fenotípica da doença. O grau de comprometimento, com base no aminoácido que substitui a glicina, pode ser representado, em linhas gerais, como $Ala \leq Ser < Cys_{red} < Arg < Val < Glu \leq Asp$ (BECK et al., 2000; BUEVICH et al., 2004).

Após o evento inicial de nucleação, a conformação em tripla hélice do colágeno tipo I é propagada da região C-terminal para a N-terminal. Assim, sugere-se que substituições de glicina ao longo da região C-terminal das cadeias α1(I) ou α2(I) resultam em fenótipos mais graves daqueles observados ao longo da região N-terminal, uma vez que atrasos no processo de dobramento da tripla hélice podem significar excessivas modificações pós-traducionais e comprometimento estrutural da molécula de colágeno nascente. A fragilidade óssea, na presença de fibrilas colágenas anormais, é o reflexo das alterações sobre as estruturas dos cristais minerais ósseos (BATEMAN et al., 1992; BUEVICH et al., 2004; BYERS; WALLIS; WILLING, 1991).

Verificar a correlação genótipo: fenótipo em OI com base nas mutações estruturais do colágeno tipo I tem sido uma tarefa bastante difícil e complexa, pois, uma dada alteração pode resultar em quadros clínicos distintos, assim como um mesmo quadro clínico pode ter origens genéticas distintas em diferentes indivíduos (MARINI et al, 2007a).

2.4 Processos Biotecnológicos Aplicados no Diagnóstico da OI

A universalização da Biotecnologia tem permitido diagnosticar diferentes doenças Mendelianas por meio de técnicas de Biologia Molecular e Bioinformática, como a Reação em Cadeia da Polimerase (PCR) e o Sequenciamento Automático, entre outros. Em virtude da grande heterogeneidade genética da doença, a associação de técnicas viabiliza a aplicação de um protocolo de diagnóstico molecular em OI. O desenvolvimento e o uso racional de biotecnologias emergentes em diagnóstico e tratamento possibilitam a qualificação de profissionais diferenciados no sentido de garantir a reprodutibilidade das técnicas empregadas para o diagnóstico molecular da doença.

2.4.1 Reação em Cadeia da Polimerase

A técnica de Reação em Cadeia da Polimerase (PCR) é um método revolucionário com base na habilidade da enzima DNA polimerase em sintetizar novas fitas de DNA complementares a uma fita molde. Pelo fato da enzima adicionar nucleotídeos somente em extremidades com o grupo 3’-OH, é necessário o uso de *primers* para que o primeiro nucleotídeo possa ser adicionado. Por este motivo, a técnica permite delinear uma região específica da sequência molde da qual se deseja amplificar. Ao

final da reação de PCR, a sequência específica será acumulada aos bilhões de cópias (amplicons) (INNIS; GELFAND, 1990; MULLIS; FALOONA, 1987; SAIKI et al., 1985).

Uma vez que não há um protocolo único apropriado para todas as situações, cada reação de PCR necessita ser padronizada a fim de se evitar produtos indetectáveis ou em baixa concentração do produto desejado, a presença de bandas inespecíficas por anelamento dos *primers* em regiões distintas, a formação de dímeros de *primers* que competem pela amplificação juntamente com os produtos desejados, entre outros. Existe uma série de parâmetros que influenciam a especificidade, fidelidade e o rendimento do produto desejado, como a concentração da enzima DNA polimerase, dNTP (deoxinucleotídeos trifosfatados), presença ou ausência de dimetilsufóxido (DMSO), concentração de $MgCl_2$ (cloreto de magnésio), nº. de ciclos e tempo/temperatura ideais em cada uma das etapas envolvidas na amplificação: (a) desnaturação do molde; (b) anelamento dos *primers* e, (c) extensão e síntese de *amplicons*, entre outros (INNIS; GELFAND, 1990; MULLIS; FALOONA, 1987; SAIKI et al., 1985).

2.4.2 Polimorfismo Conformacional de Fita Simples

A técnica de Polimorfismo Conformacional de Fita Simples (SSCP) é um procedimento simples com base na desnaturação química ou térmica de ssDNAs (DNA fita simples) de diferentes estruturas primárias que se dobram em diferentes conformações, como resultado de autocomplementaridades e de interações intramoleculares. Sob condições eletroforéticas apropriadas, *ssDNAs* de diferentes conformações migram diferencialmente durante a eletroforese em gel de poliacrilamida, e a presença de possíveis mutações pode ser detectada como um padrão de bandas com mobilidade diferenciada ou pela alteração no número de bandas visualizadas em gel (BASSAM; CAETANO-ANOLLÉS; GRESSHOFF, 1991;

FUJITA; SILVER, 1994; KALVATCHEV & DRAGANOV, 2005; ORITA et al., 1989; SPINARDI; MAZARS; THEILLET, 1991).

Da mesma forma que ocorre com a PCR, diferentes parâmetros podem influenciar a capacidade de detecção de mutações pela técnica de SSCP como o tipo de mutação presente na sequência analisada, o tamanho do fragmento de DNA e seu conteúdo de GC (guanina e citosina), a temperatura do gel durante a eletroforese, a composição e a concentração do gel, a composição do tampão de corrida (força iônica e pH), a concentração de DNA, entre outros. Em geral, diferenças em um ou mais nucleotídeos podem ser detectados em fragmentos de 100pb-300pb e 300pb-450pb com aproximadamente 99% e 90% de acurácia, respectivamente. Contudo, existem relatos de detecção de mutações em fragmentos com até 800pb (HAYASHI; YANDELL, 1993; KUKITA et al., 1997; MEUSNIER et al., 2002; SUNNUCKS et al., 2000).

2.4.3 Sequenciamento por Eletroforese Capilar

O sequenciamento é um processo que visa determinar a ordem dos nucleotídeos de um fragmento de DNA. A técnica mais utilizada é o método didesoxi ou de *Sanger*. Diferente da técnica de PCR tradicional, nesta técnica são utilizados deoxinucleotídeos (dATP, dGTP, dCTP e dTTP) e dideoxinucleotídeos (ddATP, ddGTP, ddCTP, ddTTP), que são marcados com material fluorescente e sem o grupo hidroxila no carbono 3'. Desta forma, a incorporação ao acaso de um dideoxinucleotídeo pela DNA polimerase interrompe a polimerização, o que gera ao final do processo fragmentos de tamanhos diferentes. A eletroforese capilar ou em gel dos fragmentos permite a separação por tamanho e a identificação dos fragmentos pela incidência de um laser sobre os dideoxinucleotídeos fluorescentes, o que gera um eletroferograma no qual cada nucleotídeo fica representado por um pico colorido. Ao final do processo obtemos o perfil constitutivo da sequência correspondente à região amplificada de interesse (SANGER; COULSON, 1975).

2.4.4 Next Generation Sequencing (NGS)

As tecnologias de sequenciamento evoluíram rapidamente nos últimos anos. O sequenciamento semiautomático de Sanger tem sido usado em testes clínicos por muitos anos e ainda é considerado o padrão-ouro. No entanto, suas limitações incluem baixo rendimento e alto custo, tornando análises multigênicas laboriosas e caras. As principais etapas envolvidas no sequenciamento de próxima geração (NGS) e que são genericamente aplicáveis a todas as tecnologias atuais são a escolha/construção da biblioteca, a preparação de bibliotecas e o sequenciamento paralelo massivo. O NGS pode ser utilizado no sequenciamento de genomas inteiros, ou restringir a específicas áreas de interesse, incluindo todos os 22.000 genes codificáveis (whole exome) ou um pequeno grupo de genes (BAHASSI & STAMBROOK, 2014; BEHJATI; TARPEY, 2013; KOBOLDT et al., 2013; REHM et al., 2013; YEGNASUBRAMANIAN, 2013).

Para as plataformas Illumina/Solexa Genome Analyzer e HiSeq, utilizadas em nosso estudo, as bibliotecas de DNA são clonalmente amplificadas em “bridge” para gerar clusters clonais de cada molécula de DNA *in situ* na superfície de uma “flow cell”. Essas “flow cells” são então submetidas a um sequenciamento massivamente paralelo. O fabricante Illumina (http://www.illumina.com/) utiliza uma estratégia de sequenciamento por síntese, por meio da utilização de nucleotídeos terminadores de cadeia fluorescentes reversíveis, o que permite o controle da adição de um único nucleotídeo por vez (YEGNASUBRAMANIAN, 2013).

Sule e colaboradores (2013) foram um dos pioneiros a introduzir a técnica para o estudo de genes associados a desordens esqueléticas, incluindo formas genéticas de alta e baixa densidade mineral óssea por meio de um painel contendo 21 genes, incluindo 9 genes da OI (*COL1A1, COL1A2, CRTAP, FKBP10, P3H1, PLOD2, PPIB, SERPINF1, SERPINH1* e *SP7*). Em seu estudo, a plataforma foi validada por meio da identificação de mutações em 6 pacientes com mutações já conhecidas, além de 4 pacientes sem nenhum diagnóstico molecular prévio.

2.5 Terapias Farmacológicas Empregadas no Tratamento da OI

O gerenciamento clínico da OI é um evento multidisciplinar e multiprofissional, pois envolve procedimentos cirúrgicos associados à reabilitação física progressiva, o cuidado das anormalidades auditivas, dentais e pulmonares, assim como a utilização de drogas, como os bifosfonatos e hormônio do crescimento (GH) recombinante. Os esforços terapêuticos visam maximizar a mobilidade e outras capacidades funcionais dos afetados. Em alguns casos, os atos de sentar e de caminhar são alcançados somente com o realinhamento de ossos longos, como o fêmur e a tíbia, após a aplicação de hastes intramedulares (ABULSAAD; ABDELRAHMAN, 2009; CHO et al., 2011; FORLINO et al., 2011).

No entanto, estes tratamentos não alteram a condição de fragilidade óssea, característica marcante da doença. Por esta razão, a busca por novas condutas médicas para o fortalecimento ósseo tem sido o principal foco das pesquisas em tratamento da OI. Assim, promessas futuras para o tratamento em OI envolvem o uso de terapia celular e a descoberta de novas drogas mais potentes (FORLINO et al., 2011; GLORIEUX, 2007).

Nos últimos 20 anos, uma grande variedade de bifosfonatos orais e parenterais tem sido utilizada no tratamento da OI, principalmente depois de observada uma drástica redução da dor óssea e na ocorrência de fraturas com o uso do pamidronato, um dos primeiros a ser utilizado. Os bifosfonatos são potentes agentes antirreabsortivos que inibem a função osteoclástica. A hipótese que permeia o uso destas drogas é de que a redução na atividade do sistema de reabsorção óssea possa compensar a deficiência osteoblástica (Ilustração 4). O seu uso está associado ao aumento da densidade mineral óssea (BMD), aumento no tamanho dos corpos vertebrais e espessamento do córtex ósseo, o que resulta na redução da ocorrência de fraturas e uma melhora global significativa no status funcional e deambulatório (BACHRACH; WARD, 2009; CHEUNG; GLORIEUX; RAUCH, 2009; GLORIEUX, 2007; LIN et al., 2008; LINDSAY, 2002).

Pamidronato, alendronato, risedronato, neridronato, ácido zoledrônico, olpadronato, entre outros, são bifosfonatos (análogos sintéticos do pirofosfato) que inibem a

farnesilpirofosfato sintase, uma enzima chave na via da *3-hydroxy-3methyl-glutaryl-coenzyme-A* redutase, necessária para a prenilação de proteínas intracelulares e a potencial inibição da atividade osteoclástica (CHEUNG; GLORIEUX; RAUCH, 2009; LIN et al., 2008; LINDSAY, 2002; WARD et al., 2011).

O pamidronato é um dos bifosfonatos mais utilizados nas formas grave a moderada de OI. No Brasil, a utilização do medicamento está autorizada desde 19 de dezembro de 2001, por meio da portaria nº. 2.305/GM. No Espírito Santo, o Hospital Infantil Nossa Senhora da Glória de Vitória (HINSG) é um dos centros de referência no tratamento da OI. É administrado de forma sistêmica intravenosa em ciclos de 3 dias em intervalos de 4 meses, com doses de 1mg/kg/dia de peso corporal. A terapia oral, outra forma de posologia, já foi estabelecida através do uso de olpadronato e de alendronato, com melhoras significativas no aumento da densidade mineral óssea (BMD) e redução na incidência de fraturas de ossos longos (CHEUNG; GLORIEUX; RAUCH, 2009; LIN et al., 2008; MINISTÉRIO DA SAÚDE, acesso em 27 out. 2016).

Nas formas leves da doença, é desejável que todos os pacientes apresentem níveis adequados de vitamina D (400-800U/dia) e de cálcio (800-1000mg/dia durante a infância), controlados mediante dieta ou suplementação. Contudo, deve-se avaliar a necessidade de uso de bifosfonatos e/ou hormônio do crescimento (GH) em cada caso. O GH afeta o crescimento ósseo e o *turnover* ósseo por estimular os osteoblastos, a síntese de colágeno e o crescimento longitudinal de ossos. Parâmetros referentes ao metabolismo ósseo são avaliados pelo menos duas vezes ao ano, enquanto que a massa óssea é verificada de uma a duas vezes ao ano, dependendo do comprometimento ósseo e idade do paciente, entre outros (HEATH, 2010; MONTI et al., 2010).

O ácido zoledrônico é um bifosfonato bastante recente cujo uso tem demonstrado vantagens sobre o pamidronato, tais como a aplicação em doses mais baixas (2-4mg) e menos frequentes (a cada 6 meses), por ter maior potência e eficácia a longo prazo na supressão do *turnover* ósseo e, principalmente, um baixo tempo de infusão (redução de 2-4 horas para 15 minutos), o que ocasiona uma menor irritação venosa (PANIGRAHI et al., 2010; VUORIMIES et al. 2011).

Diferentes tecnologias em Biologia Molecular têm propiciado o desenvolvimento de modelos transgênicos de OI, necessários para o desenvolvimento de terapias gênicas e celulares, como tratamentos em potencial para a OI. Contudo, um fator de complicação à terapia gênica é a heterogeneidade genética da doença e pelo fato de que grande parte das mutações em OI são dominantes negativas, onde o alelo mutado interfere no funcionamento do alelo normal (MARIJANOVIĆ et al., 2010).

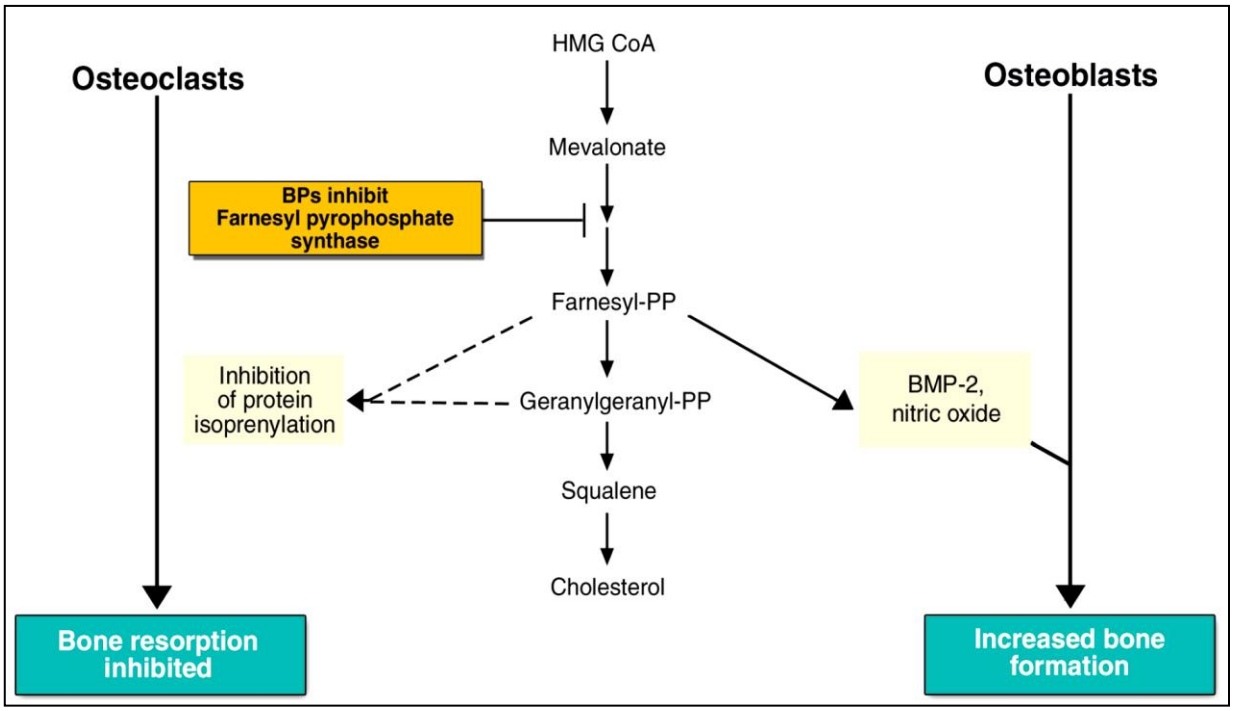


Ilustração 4 – Mecanismo Proposto de Ação dos Bifosfonatos (BP). BPs inibem um passo chave na via da *hydroxymethylglutaryl coenzyme A (HMG-CoA)* redutase através da inibição da *farnesylpyrophosphate* sintase. Inibição da isoprenilação protéica resulta na apoptose de osteoclastos e inibição da reabsorção óssea osteoclástica. *GTP=guanosine 5' -triphosphate; PP=pyrophosphate; BMP-2=bone morphogenetic protein–2* (LINDSAY, 2002).

A terapia gênica em OI visa à reposição ou o silenciamento do alelo mutante como fator de correção para o defeito causativo da doença. As terapias de supressão anti-senso visam reduzir ou o silenciar seletivamente a expressão do alelo mutante, sem interferir na expressão do alelo normal e, como consequência, transformam bioquimicamente uma condição grave da doença em uma forma mais branda. Assim, terapias com RNA de interferência (RNAi) baseiam-se no uso de pequenas moléculas de RNA dupla-fita (siRNAs) que suprimem, por sequência-dependência,

um gene expresso. Uma das limitações das técnicas anti-senso ainda é a falta de uma real especificidade contra o transcrito mutante e a dificuldade de uma expressão estável das moléculas anti-senso, o que limita a terapia gênica a estudos *in vitro* ou *ex vivo* (LINDAHL et al.,2008; MARIJANOVIĆ et al., 2010; MONTI et al., 2010).

Outra possibilidade é a reposição de células que carregam o gene mutado por células normais, obtidas a partir de células tronco mesenquimais ou embrionárias, uma vez que as mesmas podem migrar para os ossos e se diferenciarem em osteoblastos, com formação de ossos *in vivo*. Contudo, a compatibilidade doador-receptor é uma das dificuldades a ser enfrentada. Existe também a possibilidade de transplante precoce de células tronco intrauterino, durante o desenvolvimento fetal (MONTI et al., 2010).

Em suma, a combinação da terapia gênica e o transplante de células tronco estão sendo continuamente avaliada e poderá ser uma alternativa de tratamento em OI no futuro, dada a complexidade da doença (NIYIBIZI; LI, 2009).

3 OBJETIVOS

3.1 Objetivo Geral

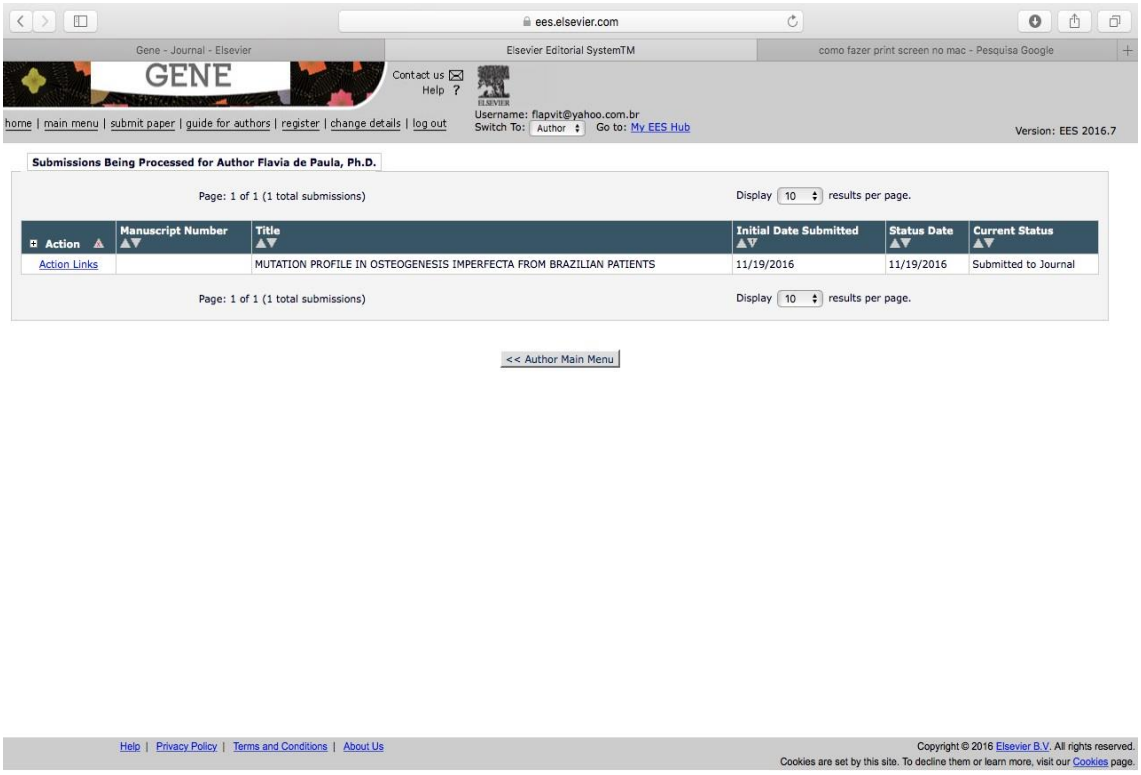
Verificar a ocorrência de alterações patogênicas em 10 genes frequentemente mutados para a Osteogênese Imperfeita e avaliar evidências clínicas existentes frente ao uso de bifosfonatos como fonte primária de tratamento farmacológico em pacientes com Osteogênese Imperfeita atendidos no centro de referência Hospital Infantil Nossa Senhora da Glória, Vitória/ES.

3.2 Objetivos Específicos

- Verificar a presença de mutações patogênicas nas formas dominantes da doença por meio da análise dos genes *COL1A1* e *COL1A2*, incluindo sítios de splicing e regiões flanqueadoras, a partir de amostras de sangue periférico extraídas de pacientes com Osteogênese Imperfeita;
- Verificar a presença de mutações patogênicas nas formas potencialmente recessivas da doença por meio da análise dos genes *CRTAP, P3H1, PPIB, SERPINF1, SERPINH1*, *FKBP10, SP7 e WNT1*, incluindo sítios de splicing e regiões flanqueadoras, a partir de amostras de sangue periférico extraídas de pacientes com Osteogênese Imperfeita;
- Verificar a correlação genótipo: fenótipo por meio da associação das mutações genéticas com os sintomas clínicos observados em pacientes.
- Avaliar a estrutura esquelética e metabolismo ósseo de pacientes com Osteogênese Imperfeita (OI) em resposta ao uso de pamidronato dissódico.

4 ARTIGOS CIENTÍFICOS DERIVADOS DA TESE

4.1 Manuscrito 1 - Mutation Profile in Osteogenesis Imperfecta from Brazilian Patients



MUTATION PROFILE IN OSTEOGENESIS IMPERFECTA FROM BRAZILIAN PATIENTS

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ABSTRACT

Osteogenesis Imperfecta (OI) is a genetic heterogeneous disorder characterized by bone fragility and fracture. Mutations in 19 distinct genes can cause Osteogenesis Imperfecta, and therefore becomes the molecular diagnosis of OI is frequently difficult to be analyzed and expensive. Studies that report the most frequently muted genes in OI patients can help to improve molecular strategies of diagnosis for the disease. In order to characterize the mutation profile of OI in Brazilian patients, we analyzed 31 unrelated patients though Single Strand Conformation Polymorphism (SSCP) screening, Next Generation Sequencing (NGS) and/or Sanger Sequence for the ten most frequently mutated genes on database of mutations, including: *COL1A1*, *COL1A2, P3H1*, *CRTAP*, *PPIB*, *SERPINH1*, *SERPINF1*, *FKBP10, SP7 and WNT1* genes. Disease-causing variants were identified in 30 cases. Twenty-four patients (77,41%) carry mutations on *COL1A1* or *COL1A2* genes and six patients (19%) carries genetic changes on *P3H1* or *FKBP10* gene. Moreover, a total of 28 distinct mutations were identified, including 11 novel changes. In one case, the causative mutation was not detected. No mutations were found on *CRTAP*, *PPIB*, *SERPINH1*, *SERPINF1*, *SP7* and *WNT1* genes, suggesting that causative mutations on these genes are rare. However, it has to take in considerations that distinct populations can have different proportion of disease-causing variants. Hence, is important to replicate this study in other groups. In conclusion, our data show that the analysis of the *COL1A1*, *COL1A2*, *P3H1* and *FKBP10* genes is able to detect up to 95% of causative-mutations in Osteogenesis Imperfecta disorder.

KEYWORDS: NGS; Osteogenesis Imperfecta; *COL1A1* gene; *COL1A2* gene; *P3H1* gene; *FKBP10* gene.

LIST OF ABBREVIATIONS

INDELs - Insertions/Deletions
NGS - Next Generation Sequence
OI - Osteogenesis Imperfecta
SNVs - single-nucleotide variants
SSCP - Single Strand Conformation Polymorphism

1. INTRODUCTION

Osteogenesis Imperfecta (OI) is a heterogeneous group of connective tissue syndromes characterized by abnormal bone fragility that leads to fractures and skeletal deformities. The prevalence of OI is estimated in 1/15,000 (Marini et al., 2010; Sillence et al., 1979; Van Dijk et al., 2010). Because of the wide clinical variability, patients can also develop short stature, dentinogenesis imperfecta, blue sclera and hearing loss. The phenotypic spectrum of OI may overlap with others skeletal diseases, which makes the establishment of a precise diagnosis based on clinical, radiological and genetic investigation extremely difficult. Based on clinical diagnosis, including pre-and postnatal severity of bone fragility, the traditional classification distinguishes four phenotypic groups (OI types I to IV). The OI type I is the mildest phenotype, OI type II is lethal in neonatal period, OI type III is the most severe form compatible with postnatal survey and the OI type IV represents a moderate form of severity. However, it has been suggested an expanded classification based on phenotype and mutated gene (Sillence et al., 1979; Van Dijk and Sillence, 2014).

OI-like syndromes with a dominant or recessive pattern of inheritance can be associated up to nineteen genes (Dalgleish, 1998, 1997; Van Dijk and Sillence, 2014). A large proportion of OI cases that segregate in an autosomal dominant pattern are due to heterozygous mutations in the structural genes coding for the two procollagen chains *COL1A1* [MIM 120150] and *COL1A2* [MIM 120160] from type I collagen structure, the main protein of bone, tendons and cartilage (Van Dijk et al., 2010). In addition, mutations in *IFITM5* [MIM 614757], the gene encoding BRIL, a transmembrane protein enriched in osteoblasts during mineralization, are less frequently but are also related (Farber et al., 2014; Semler et al., 2012).

Unlike the autosomal dominant pattern of inheritance, those individuals who do not have a mutation in one of the structural collagen genes might have recessive mutations in others loci. The number of genes discovered that may lead to recessive phenotypes have increased dramatically, resulting in a total of sixteen loci, others than *COL1A1* and *COL1A2* (Dalgleish, 1998, 1997; Martínez-Glez et al., 2012; Symoens et al., 2013). Among them, there are important genes as *CRTAP* [MIM 605497], *P3H1* [MIM 610339], *PPIB* [MIM 123841], *SERPINH1* [MIM 600943], *FKBP10* [MIM 607063], *SERPINF1* [MIM 172860] and *SP7/OSX* [MIM 606633]. The *CRTAP P3H1* and *PPIB* genes encode the 3-hydroxylation complex of Pro 986 in the proα1(I) (Barnes et al., 2006; Cabral et al., 2007; Ishikawa et al., 2009; Morello et al., 2006; Fleur S Van Dijk et al., 2009; Fleur S. Van Dijk et al., 2009). The *SERPINH1* and *FKBP10* genes are chaperons involved in type I procollagen formation (Alanay et al., 2010; Christiansen et al., 2010; Drögemüller et al., 2009; Duran et al., 2014; Zhou et al., 2014). The *SERPINF1* gene encodes a type I collagen interacting protein and antiangiogenic extracellular matrix factor (Al-Jallad et al., 2014; Becker et al., 2011) while *SP7/OSX* gene encodes a protein involved in cell differentiation (Lapunzina et al., 2010).

According to the best practice guidelines of laboratory diagnosis for OI, Sanger sequencing of relevant genes is the gold standard (Van Dijk et al., 2012). Given the large number of suspected genes linked to OI and the phenotypic heterogeneity of it, Sanger sequencing of various genes might increase the sensitivity of molecular diagnosis of a disease. However, is not only expensive but also laborious and a very time-consuming procedure. The emergence of new technologies

for high-throughput capture and the possibility of the inclusion of a maximum number of genes into a sequencing panel has made the Next-Generation Sequencing (NGS) one of the most promising techniques for molecular diagnosis purposes (Árvai et al., 2016; Sule et al., 2013).

Herein, we present the molecular results from 31 individuals with typical OI phenotype, obtained by the use of the SSCP screening, NGS techniques or Sanger Sequencing for the identification of the mutation profile in the ten genes most often mutated in Osteogenesis Imperfecta patients, that include the *COL1A1*, *COL1A2*, *P3H1*, *CRTAP*, *PPIB*, *SERPINH1*, *SERPINF1*, *FKBP10*, *SP7 and WNT1* genes.

2. MATERIALS AND METHODS

2.1 SUBJETS

A total of 31 patients with clinical and radiological diagnosis of OI were included in this work. All patients were selected from Hospital Estadual Infantil Nossa Senhora da Glória (HINSG), Vitória-ES, Southeast of Brazil, with approval by the Ethics Committee. The participants of this study were recruit from 2006 to 2012. All participants have given informed consent to participate in this study. The P.1, P.11, P.12, P.28, P.6, P.13, P.25, P.26 and P.27 patients were previously described by our group (Barbirato et al., 2009, 2015, 2016; Moraes et al., 2012).

2.2 MUTATIONAL ANALYSIS

The Osteogenesis Imperfecta Variant Database contains a compilation of genetic variants from 19 OI-related genes (Dalgleish, 1998, 1997). In the present work we selected the *COL1A1*, *COL1A2*, *P3H1*, *CRTAP*, *PPIB*, *SERPINH1*, *SERPINF1*, *FKBP10, SP7 and WNT1* genes. Our choice was based in those genes reported as the most prevalent in causative-OI mutation (Dalgleish, 1998).

DNA sample was collected from periphery blood and extracted by Miller protocol (1988). PCR of exons and exon/intron boundaries were done in genomic DNA following by Single Strand Conformation Polymorphism (SSCP) screening and sequencing of abnormal fragments on polyacrylamide gel for the analysis of *COL1A1*, *COL1A2*, *P3H1*, *CRTAP*, *PPIB*, *SERPINH1*, *SERPINF1* and *FKBP10* genes until 2013. As the NGS technology can detect genetic variations with higher sensibility than SSCP screening, we re-analyzed patients that the molecular diagnosis was not conclusive in May 2015 through NGS panel that contained the *COL1A1*, *COL1A2*, *P3H1*, *CRTAP*, *PPIB*, *SERPINH1*, *FKBP10 and SP7* genes. After July 2015 patients without conclusive molecular results were analyzed through Direct Sanger Sequencing for *WNT1* gene.

For SSCP screening, all exons of analyzed genes and their flanking regions were screening by using 5-7% acrylamide gels and commercial version MDE® Mutation Detection Enhancement Gel (Lonza Group Ltd., USA) to improve

sensitivity. Fragments with abnormal pattern on SSCP gels were analyzed after silver staining and Sanger sequencing was performed in ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA). Restriction Enzyme or Allele-Specific PCR was used to validate the mutations identified through SSCP screening.

For the NGS methodology the technique was performed with kit NEXTERA (Illumina, USA) that was used to prepare the samples bibliotheca and genes capture. A total of 10 ng DNA/sample were used for the target enrichment step. To quantification of the samples and to verify the length of the bibliotheca we used the High Sensivity DNA kit in the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). qPCR was performed by KAPA Library Quantification Kit in the Real Time LightCycler® System (Roche, DE). The captured libraries were sequenced with Miseq Sequencer (Illumina, USA).

NGS sequence reads were aligned to the human reference genome (hg19, GRCh37) with the Burrows-Wheeler Aligner (BWA, version 0.6.1) (Li and Durbin, 2009). To verify single-nucleotide variants (SNVs) substitutions and small indel (INDELs), variants were called with SAM tools (version 0.1.18), Picard tools (version 1.60) and a Genome Analysis Toolkit (GATK, version 1.5.21) (Li et al., 2009; McKenna et al., 2010) that also settled the SNVs list. Genotypes were called at all positions with high-quality sequence bases and filtered to retain SNVs and insertion-deletions with Phred-like quality scores of at least 20.

3. RESULTS

This study comprised a total of 31 (16 males; 15 females) unrelated OI patients, based on clinical and radiological diagnosis. According to the Sillence's (1979) classification, OI type III (severe) were diagnosed in 38,70% of the patients (12/31), OI types IV (moderate) in 22,50% of the sample (7/31) and OI type I (mild) in 38,70% (12/31) of the total. The OI type II (lethal) cases were not included in this work. Isolated OI cases accounted for 64,52% (20/31) of the sample. Additionally, positive familial history was reported by 32,26% (10/31) of the patients. There were affected members in more than one generation according the autosomal dominant pattern of inheritance in eight cases. Also, it was observed 2 cases that only affected sibs, suggesting autosomal recessive pattern. The assessment of familial history was not available for patient P.12. Only one patient (P.2) reported consanguinity of their parents.

Disease-causing variants were identified in 96,77% (30/31) of the study population. The initial SSCP screening and Sanger Sequencing of abnormal fragments on polyacrylamide gel allowed the detection of mutations in 12 cases, including 7 mutations on *COL1A1* gene, 1 change on *COL1A2* gene, 3 genetic variants on *P3H1* gene (1 known mutation and two likely pathogenic variants) and 1 mutation on *FKBP10* gene. In the following analysis, the NGS methodology was performed in 22 patients that the result was not conclusive in the previous analysis. The NGS technique allowed the identification of mutations in 21 patients, including 8 pathogenic changes on COL1A1 gene, 9 mutations on COL1A2 gene and 2 pathogenic variants on *FKBP10* gene. The NGS methodology also allowed the confirmation of the likely pathogenic variants previously detected by SSCP following by Sanger Sequencing in two patients on *P3H1* gene. In one case, the causative OI mutation was not detected. Overall, a total of 28 distinct mutations were identified, including 11 novel changes. Three variants were

reported twice in distinct families. The remaining 24 genetic variants were unique. In the total, 13 mutations were found on *COL1A1* gene and 9 on *COL1A2* gene. Mutations on *P3H1* gene were detected in 3 patients. Other 3 unrelated cases carry mutations on FKBP10 gene. No mutations were found on *CRTAP, PPIB, SERPINF1, SERPINH1, WNT1* and *SP7* genes in the studied sample. The main results are listed in Table 1.

4. DISCUSSION

In our study, we analyzed 31 unrelated OI patients for *COL1A1*, *COL1A2, P3H1*, *CRTAP*, *PPIB*, *SERPINH1*, *SERPINF1, FKBP10, SP7 and WNT1* genes through SSCP screening, NGS techniques or Sanger Sequencing. We identified pathogenic mutations in 96,77% of the sample, including 11 novel changes. Causative OI mutations were detected in 77,41% of genes related with autosomal dominant OI (*COL1A1* or *COL1A2* genes) and 19% among genes involved with autosomal recessive OI forms (*P3H1* or *FKBP10* genes). Since there are different genes related with OI and the lack of hot spot of mutations in most of populations, the investigation of mutations becomes expensive and difficult.

In the present work the selection of *COL1A1*, *COL1A2, P3H1*, *CRTAP*, *PPIB, SERPINH1*, *SERPINF1*, *FKBP10, SP7 and WNT1* genes among others 19 distinct genes that can cause OI allowed the detection of genetic changes up to 95% of the sample, supporting the idea in which these genes share most of mutation among OI patients. We failed to identify mutation in one case. It occurs probably because this patient carries pathogenic changes in one gene that was not analyzed in this study or because the pathogenic variant was in a regulatory region that was not studied. When a causative OI mutation cannot be identified in the mainly genes studied, the others genes must be analyzed to define the molecular diagnosis.

Bardai, et al. (2016) proposed the division of OI patients in groups according to their phenotype to improve the identification of causative OI-mutation. In our sample, when we divided the patients using this parameter, all cases with mild spectrum in which the mutation was identified showed mutation on COL1A1/COL1A2 genes, as previously described (Bardai et al., 2016). However, the proportion of causative-OI mutations changes among the group of moderate/severe phenotype. In our sample 80% (24/30) of patients carry pathogenic changes in *COL1A1/COL1A2* genes and 20% (6/30) carry mutations on *P3H1* or *FKBP10* genes. Bardai et al. (2016), in a study enrolled 598 OI individuals, showed that 77% of patients with moderate/severe spectrum carry mutations on COL1A1/COL1A2 genes, 9% of the cases share genetic changes on *IFITM5* gene, and 12% of patients carry mutations on genes related with recessive OI pattern. Bardai et al. (2016) showed that the genes with more prevalent number of mutations in their study among those with recessive OI pattern are *SERPINF1, CRTAP, P3H1, WNT1* and *FKBP10* genes, respectively. In our study, we identified pathogenic changes only in *P3H1* and *FKBP10* genes.

Several works suggest that pathogenic changes on *COL1A1/COL1A2* genes are observed in about 90% of OI patients (Árvai et al., 2016). Bardai et al. (2016) discuss that this percentage was estimated in the past when only mutations on *COL1A1/COL1A2* genes were knowledge. They concluded that the identification of other genes related with OI become the proportion of mutations on *COL1A1/COL1A2* more reliable. Their work suggest that about 86% of mutations are due

mutations on *COL1A1* or *COL1A2* genes. In our study, changes on these genes are responsible by 77% of the sample. Overall, according Bardai et al. (2016) and our study, the data suggest that the number of mutations on *COL1A1/COL1A2* genes probably varies between 75 to 85% among OI patients in different populations.

The majority of causative-OI mutations were identified in the *COL1A1* gene, representing 50% (15/30) of all variants detected in this study, following by 30% (9/30) from *COL1A2* gene, 10% (3/30) from *P3H1* gene and 10% from *FKBP10* gene (10/30). As reposted in several works, one half or more of OI patients carry changes on *COL1A1* gene, following by *COL1A2* gene (Bardai et al., 2016; Ho Duy et al., 2016; Lindahl et al., 2015; Zhang et al., 2012). Moreover, our data also confirmed that about 60% of mutations on *COL1A1/COL1A2* gene were glycine substitution, while 40% of patients carry other type of genetic variations, including mainly change on splice site and frameshift mutations.

Four novel changes were identified on COL1A1 gene: two missense and two frameshift mutations. The *COL1A1* c.887G>T (p.Gly296Val) and c.2165G>C (p.Gly722Ala) changes were found in the unrelated P.2 and P.7 patients from mild OI group. Both variation cause triple-helical glycine substitutions, within exons 13 and 32, respectively. These particular changes appear to be related to mild OI phenotypes, as seen in our sample. However, other missense mutations involving substitutions for glycine in these exons have been related to a wide spectrum of OI, including lethal and non-lethal outcomes (Dalgleish, 1998, 1997). The *COL1A*1 c.2750delG and c.3239delC mutations were found in three unrelated patients from moderate to severe OI group (P.10, P.11, and P.15). These frameshift variants affects exons 40 (p.Gly917Aspfs*191) and 45 (p.Pro1080Leufs*28), located within proa1(I)-C-propeptide, respectively. Similar changes in these exons were in general linked to mild OI and moderate to severe OI (Pyott et al., 2011).

Two novel mutations were identified on COL1A2 gene. The c.3296G>C (p.Gly1099Ala) at proa2(I)-C-propeptide cause a triple-helical glycine substitution which affects the stability of the collagen trimer and thus leading to a OI with severe phenotype (P.24), as seen in our sample. A similar change affecting the same glycine has already described. The c.3295G>A p.(Gly1099Arg) changes the obligatory glycine for arginine and also results into a severe OI spectrum (Faqeih et al., 2009; Marini et al., 2010). The c.2207G>T (p.Gly736Val) change, found in patient P.10, also changes a obligate Glycine residue on proa2(I)-C-propeptide.

The *COL1A2* c.2207G>T (p.Gly736Val) mutation was found in patient P.10, who also presented the *COL1A1* c.2750delG frameshift change. This case appears to be the first biallelic mutations reported occurring in COL1A1 and COL1A2 genes, simultaneously. The similar c.2206G>T [p.Gly736Cys] mutation, affecting the same glycine, has already described occurring in OI with mild and moderate phenotype (Dalgleish, 1998, 1997). Mackenroth et al. (2016) described an overlapping phenotype of Osteogenesis Imperfecta and Ehlers-Danlos syndrome (EDS). This outcome was due to the heterozygous *COL1A1* c.4006-1G>A mutation and the c.3637G>A (p.Val1213Val) and c.7774G>A (p.Gly2592Ser) biallelic missense variations in *TNXB* gene. The analysis of cDNA showed that the *COL1A1* splice-site variant leads to retention of intron 50 which contains a premature stop codon (p.Phe1336Valfs*72), thus causing a frameshift (Mackenroth et al., 2016). The patient presents features that are compatible with mild OI and hypermobility type EDS (Mackenroth et al., 2016).

Genes involved with recessive OI forms of inheritance that were previously reported by our group (Barbirato et al., 2016, 2015) were re-analyzed in this research. NGS technology confirmed what was predicted as disease-causing c.2024G>T (p.Trp675Leu) missense change on *P3H1* gene in heterozygous state in P.27 patient. The NGS methodology also confirmed the c.1087A>G (p.Lys363Glu) missense change in heterozigosity in P.26 patient. In this case, the mutation was predicted as a benign change *in silico* study (Schwarze et al., 2013), however no functional study was done to analysis the relevance of this change in clinical spectrum. In both cases, no other genetic change was found in none of studied genes. We did not observed these two variants in approximately 600 non-affected OI individuals analyzed by NGS methodology. Probably the second pathogenic change in these patients is localized in a non-coding region of the *P3H1* gene that was not analyzed in this work. However, as we did not find the second change in these two patients, we cannot exclude the fact that their sequencing results are inconclusive.

The use of NGS technique allowed the identification of the two variants in a compound heterozygous state in the P.29 patient, the c.179A>C (p.Gln60Pro) missense change (exon 1) and the c.1063+2T>C splicing site alteration (intron 6) on *FKBP10* gene. Other change identified in the same gene by NGS methodology was the c.21dupC homozygosity mutation detected in P.28 patient. All these mutations on *FKBP10* gene are predicted as pathogenic changes.

The *FKBP10* c.831dupC frameshift change and the *P3H1* c.1080+1G>T mutation, previously identified by our group in (Barbirato et al., 2016, 2015, respectively), are very well described in the literature. Therefore, they were re-analyzed only by Sanger Sequence methodology. These homozygous changes were confirmed in the patients P.30 and P.25, respectively. The *P3H1* c.1080+1G>T change has carrier frequency of about 1/240 in the African American population and usually results in a perinatal lethal form of OI (Cabral et al., 2007). In some populations, the carrier frequency of this allele is relatively high. As the carrier frequency in a population increases, the proportion of infants with recessively inherited OI due to homozygosity for one allele increases, as demonstrated in homozygosity for *P3H1* c. 1080+1G>T in West African and the presence of founder mutations in other distinct geographic endogamous group (Pepin et al., 2013). In our sample, this change was observed only once among 31 unrelated OI patients. The number of studied patients for this rare change in our work is small to infer their approximated proportion in our population.

The great number of techniques involving sequence of whole genome becomes a challenge to characterize the clinical status of variants with uncertain significance (VUS). The characterization of different VUS among distinct groups can help the interpretation of clinical spectrum of rare variants. In the present study we re-analysis through NGS technique the status of six genetic variants: i) the c.1812C>T (p.Pro604=) synonymous variant on *P3H1* gene; ii) the c.558A>G (p.Ala186=) change on *CRTAP* gene; the two following changes present on *FKBP10* gene: iii) the c.590A>G (p.Lys197Arg) missense change; and iv) c.1546G>A (p.Leu516Phe) variant; and the two following changes on *SERPINF1* gene: v) the c.18A>G (p.Leu6=) synonymous variation and vi) c.21C>A (p.Leu7=) change, previously reported by our group (Barbirato et al., 2016, 2015). These variations are present in P.1, P.11, P.12 and P.28 patients that carry pathogenic known mutations on *COL1A1* or *FKBP10* genes. The identification of known pathogenic mutations in patients that carry these genetic variants suggests that these changes are rare non-pathogenic variants.

Although we selected the genes with major frequency of mutation causing the disease among those related with dominant and recessive pattern, some genes, as *IFITM5* gene, were not analyzed in this work. The frequency of disease-causing variants in this gene related with the dominant inheritance can vary a lot among different populations and might represent about 5% of autosomal dominant OI cases. This gene has a hot spot mutation, an atypical characteristic among those genes related with OI disease (Hoyer-Kuhn et al., 2014). We cannot disregard that our patients who we did not found OI causative mutations can carry pathogenic changes on this gene.

The presence of high consanguinity or founder effect can change the prevalence of mutations among different populations, as described by Bardai et al. (2016), that found major number of mutations on *SERPINF1* and *CRTAP* genes among those related with recessive pattern. Minilo et al. (2014) also found the same mutation on *SERPINF1* gene in two unrelated families, one of them with at least 4 generations with affected patients, supporting the hypothesis of founder effect. In our work, only one family reported consanguinity. In our study, mutations were not identified neither on these genes nor on *PPIB*, *SERPINH1*, *WNT1* and *SP7* genes, suggesting that mutations on these genes are rare in studied sample.

The identification of mutated genes and the differentiation between dominant and recessive autosomal forms in patients can provide the basis for an accurate counseling regarding prognosis and reproductive purposes. Mostly, to provide psychological benefits to patients and their families, thus allowing the prevention of new cases of the disease in the population. Moreover, an accurate molecular postnatal diagnosis may help initiate appropriated therapeutic interventions in those who the clinical progression of the disease can be improved by bisphosphonate therapies. The bisphosphonate is a drug that increases bone mineral density and reduces the number of fractures and bone pain. So far, the combination of next-generation sequencing technology (NGS) and bioinformatics has significant impact on clinical care of patients. They constitute a powerful tool for discovery of causative mutations in rare Mendelian disease genes, considering the discovery of new genes and the limited genotype-phenotype correlation related to OI disease.

In this study, we analyzed 31 unrelated OI patients through mutations in the ten most frequently mutated genes from mutations OI database. Causative changes were found in almost 97% of the cases, including 50% on *COL1A1 gene*, 33% on *COL1A2* genes, 10% on *P3H1* gene and 10% on *FKBP10* gene. We did not detect a disease-causing variant in one patient. Our data support the hypothesis that mutations on *COL1A1* or *COL1A2* are responsible for about 83% of pathogenic changes in OI cases. In our study, most patients without mutations on *COL1A1/COL1A2* genes carry pathogenic changes on *P3H1* or *FKBP10* genes. This suggests that these genes have major number of genetic changes among those with recessive pattern. The molecular diagnosis of OI becomes extremely difficult due the presence of 19 distinct genes related and numerous exons on some of these genes, as in *COL1A1* and *COL1A2* genes. Hence, the study of most frequently muted genes in OI patients can help improve molecular strategies of diagnosis for the disease. Our data show that the analysis of these four genes in OI patients is able to detect up to 95% of causative-mutations. We cannot forget that distinct populations can have different proportion of disease-causing variants. Therefore, the replication of this study in other groups is necessary for knowledge of profile of OI mutations in distinct communities.

5. CONCLUSION

Our data suggest that the analysis of the *COL1A1*, *COL1A2*, *P3H1* and *FKBP10* genes was able to detect up to 95% of causative-mutations in Osteogenesis Imperfecta disorder from Brazil.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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AUTHOR'S CONTRIBUTIONS

The principal researchers of the work were Marcos Vinícius Dornelas de Moraes, Maira Trancozo and Flavia de Paula. Marcos Vinícius Dornelas de Moraes, Clara Barbirato and Márcio Germello de Almeida obtained biological samples and clinical data of the participants. Marcos Vinícius Dornelas de Moraes, Maira Trancozo, Meire Aguena and Dalila Avila Silva done the experimental procedures. Maria Regina Galveas Oliveira Rebouças, Akel Nicolau Akel Jr, Valentim Sipolatti and Vanda Regina Rangel Nunes helped with the clinical assessment of the patients. Marcos Vinícius Dornelas de Moraes, Maira Trancozo and Flavia de Paula wrote the manuscript. Flavia Imbroisi Valle Errera, Lígia Ramos dos Santos and Maria Rita Passos Bueno gave support for experiments procedures and helped with suggests for the manuscript discussion. All authors review the manuscript.

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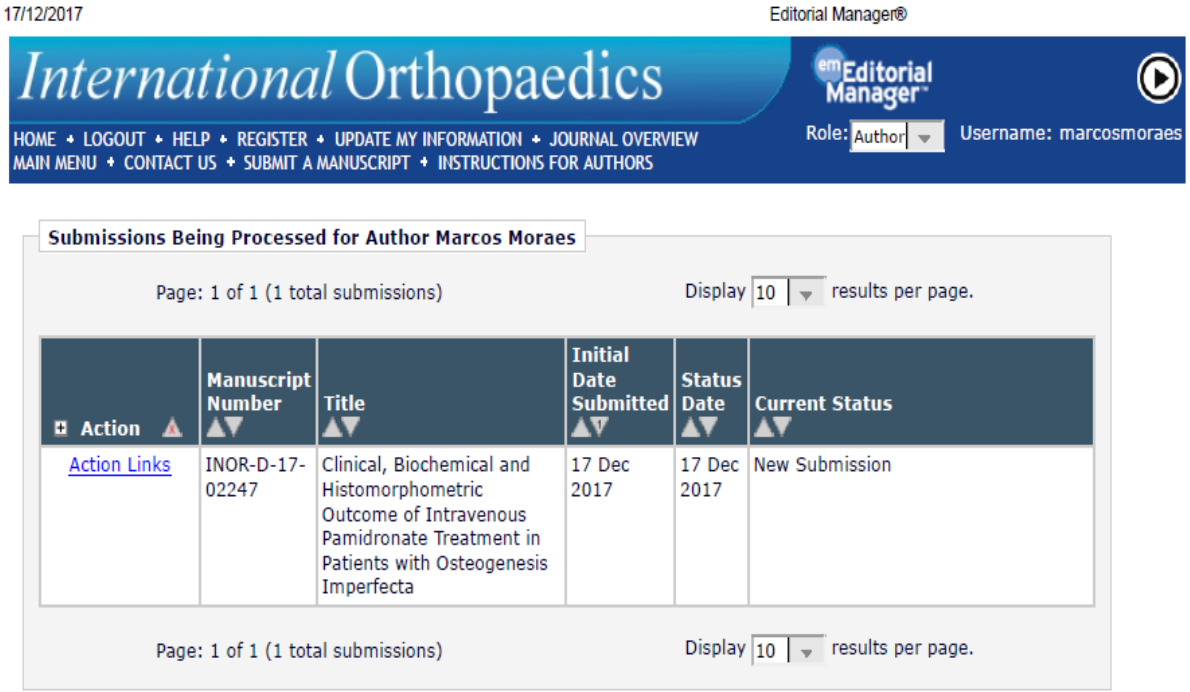
Table 1. Genetic variations found in Osteogenesis Imperfecta patients.

Patient	Gene	Exon/Intron	Mutation (cDNA)	Mutation (Protein)	Mutation Type	Zygosity	OI Type	Familial	Prediction	Novel
P1	COL1A1	Exon 8	c.608G>T	p.Gly203Val	Missense	Heterozygous	III	No	Pathogenic	No
P2	COL1A1	Exon 13	c.887G>T	p.Gly296Val	Missense	Heterozygous	I	Yes	Pathogenic	Yes
P3	COL1A1	Intron 16	c.1056+1G>A	Splice Site	Splicing	Heterozygous	IV	No	Pathogenic	No
P4	COL1A1	Exon 17	c.1138G>T	p.Gly380Cys	Missense	Heterozygous	I	Yes	Pathogenic	No
P5	COL1A1	Exon 18	c.1165G>T	p.Gly389Cys	Missense	Heterozygous	IV	No	Pathogenic	No
P6	COL1A1	Intron 27	c.1875+1G>C	Splice Site	Splicing	Heterozygous	III	No	Pathogenic	No
P7	COL1A1	Exon 32	c.2165G>C	p.Gly722Ala	Missense	Heterozygous	I	No	Pathogenic	Yes
P8	COL1A1	Exon 36	c.2523delT	p.(Gly842Alafs*266)	Frameshift	Heterozygous	I	Yes	Pathogenic	No
P9	COL1A1	Intron 37	c.2559+1G>A	Splice Site	Splicing	Heterozygous	I	Yes	Pathogenic	No
P10	COL1A1	Exon 40	c.2750delG	p.(Gly917Aspfs*191)	Frameshift	Heterozygous	III	Yes	Pathogenic	Yes
P11	COL1A1	Exon 40	c.2750delG	p.(Gly917Aspfs*191)	Frameshift	Heterozygous	III	Yes	Pathogenic	Yes
P12	COL1A1	Exon 43	c.3162delT	p.(Gly1055Alafs*53)	Frameshift	Heterozygous	I	N/A	Pathogenic	No
P13	COL1A1	Exon 45	c.3235G>A	p.Gly1079Ser	Missense	Heterozygous	I	Yes	Pathogenic	No
P14	COL1A1	Exon 45	c.3235G>A	p.Gly1079Ser	Missense	Heterozygous	I	Yes	Pathogenic	No
P15	COL1A1	Exon 45	c.3239delC	p.(Pro1080Leufs*28)	Frameshift	Heterozygous	III	No	Pathogenic	Yes
P16	COL1A2	Intron 14	c.693+2T>C	Splice Site	Splicing	Heterozygous	IV	No	Pathogenic	No
P17	COL1A2	Exon 16	c.739G>C	p.Gly247Arg	Missense	Heterozygous	IV	No	Pathogenic	No
P18	COL1A2	Exon 37	c.2027G>A	p.Gly676Asp	Missense	Heterozygous	I	No	Pathogenic	No
P10	COL1A2	Exon 37	c.2207G>T	p.Gly736Val	Missense	Heterozygous	III	Yes	Pathogenic	Yes
P19	COL1A2	Exon 38	c.2314G>A	p.Gly772Ser	Missense	Heterozygous	I	No	Pathogenic	No
P20	COL1A2	Exon 38	c.2314G>A	p.Gly772Ser	Missense	Heterozygous	I	Yes	Pathogenic	No
P21	COL1A2	Intron 40	c.2565+1G>A	Splice Site	Splicing	Heterozygous	IV	No	Pathogenic	No
P22	COL1A2	Exon 40	c.2458G>A	p.Gly820Ser	Missense	Heterozygous	III	No	Pathogenic	No
P23	COL1A2	Exon 49	c.3278G>A	p.Gly1093Asp	Missense	Heterozygous	III	No	Pathogenic	No
P24	COL1A2	Exon 49	c.3296G>C	p.Gly1099Ala	Missense	Heterozygous	III	No	Pathogenic	Yes
P25	P3H1	Intron 5	c.1080+1G>T	Splice Site	Splicing	Homozygous	III	No	Pathogenic	No
P26	P3H1	Exon 6	c.1087A>G	p.Lys363Glu	Missense	Heterozygous	III	No	Benign	No
P27	P3H1	Exon 14	c.2024G>T	p.Trp675Leu	Missense	Heterozygous	III	Yes	Pathogenic	No
P28	FKBP10	Exon 1	c.21dupC	p.Ser8Glnfs*67	Frameshift	Homozygous	IV	No	Pathogenic	No
P29	FKBP10	Exon 1 Exon X	c.179A>C c.1063+2T>C	p.Gln60Pro Splice Site	Missense Splicing	Heterozygous Heterozygous	IV	No	Pathogenic Pathogenic	Yes Yes
P30	FKBP10	Exon 5	c.831dupC	p.Gly278Argfs*95	Frameshift	Homozygous	III	No	Pathogenic	No
P31	-	-	-	-	-	-	I	No	-	-

N/A: Not available

4.2 Manuscrito 2 - Clinical, Biochemical and Histomorphometric Outcome of Intravenous Pamidronate Treatment in Patients with Osteogenesis Imperfecta



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CLINICAL, BIOCHEMICAL AND HISTOMORPHOMETRIC OUTCOME OF INTRAVENOUS PAMIDRONATE TREATMENT IN PATIENTS WITH OSTEOGENESIS IMPERFECTA

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ABSTRACT

In this retrospective study, the effects of cyclic intravenous pamidronate disodium protocol in patients with osteogenesis imperfecta (OI) are investigated. Forty patients with OI who were seen at the Hospital Nossa Senhora da Glória (HINSG) underwent a cyclic intravenous pamidronate disodium protocol. To evaluate the pamidronate disodium efficacy in the histomorphometric outcomes, number of fractures, skeletal structure alterations, bone pain and bone formation/resorption process, samples and data were collected. Bone densitometry and biochemical examinations were performed periodically in patients undergoing treatment. Bone pain was assessed using a visual analogue linear scale. In conclusion, an increase in bone mass after intravenous pamidronate infusion, with consequent decrease in fracture rates, improvement of bone pain and patient mobility is expected. The decrease in C-telopeptide and serum alkaline phosphatase values confirm the reduction of bone turnover after starting treatment. Long-term intravenous bisphosphonate therapy was associated with higher Z-scores for lumbar spine and bone mineral density.

KEYWORDS

osteogenesis imperfecta; bisphosphonate; bone mineral content; bone mineral density; bone fragility; bone fractures

INTRODUCTION

Osteogenesis imperfecta (OI) is a heterogeneous genetic disease mainly caused by mutations in Type I collagen genes. With an incidence of approximately 1: 10,000 individuals, it is characterized by bone fragility, low bone mineral density (BMD) and recurrent fractures with progressive deformity and consequent mobility limitation. Additional features as blue sclera, dentinogenesis imperfecta, skin hyperelasticity and hypermobility of joints are common [1]. The clinical problem of this disease lies on an inadequate bone development that leads to fractures, growth failure and even death in the most severe phenotypes.

Based on their clinical, radiological and genetic characteristics, Sillence's classification consists of four classical OI types. Type I is the mildest form of the disease and includes patients without significant bone deformities. Type II is generally lethal in the perinatal period due to multiple intrauterine fractures and severe bone deformity. Type III is the most severe form in children who survive after the neonatal period, as these patients have marked short stature and deformities in the limbs and spine due to multiple fractures. Type IV includes patients with moderate bone deformity, short stature and a very variable phenotype. Histological and biochemical studies of the bones of patients with OI showed an increase in osteoclastic activity and a reduction in the formation of new bone, contributing to diminished bone mineral density [1, 2].

Many pharmacological treatments affecting various aspects of bone metabolism have been studied as potential medical therapies for OI, such as fluorides, calcitonin, growth hormone (GH), anabolic steroids and magnesium oxide, however, it has not been possible to show any consistent beneficial effect [3]. Until now, pamidronate disodium has been largely used because its beneficial effect in children and adolescents with different forms of OI, thus, increasing bone mineral density in areas of bone turnover, decreasing fracture rates, and promoting bone pain and mobility improvement. The aim of this study was to evaluate the effects of this drug in OI patients and associated outcomes.

MATERIALS AND METHODS

This study evaluated the evolution of OI patients, who were seen at the Hospital Nossa Senhora da Glória (HINSG), located in the city of Vitória/ES, by analyzing the efficacy of the drug in the reduction of fracture numbers and bone pain, as well as skeletal changes and bone metabolism. We also analyzed the degree of mobility and the evolution of growth in weight and height of children with OI during the treatment period compared to the standard growth curves (developed by the National Center for Health Statistics in 2000, www.cdc.gov/growthcharts) from CDC (Center of Disease Control). Through a comparative analysis of each bone densitometry performed annually in patients undergoing treatment, we can analyze the effect of pamidronate on the increase of bone mineral density, taking as parameters the values of bone mineral density (BMD), bone mineral content (BMC) and z-score of the lumbar spine (L1-L4) and of the whole body. The changes caused by pamidronate in biochemical markers of bone metabolism - substances that depict bone formation or resorption - were also evaluated through the results of biochemical tests taken on the first day of the cycle - prior to the

administration of pamidronate - and in the 3rd day after the medication. From the data obtained, the following parameters were analyzed: alkaline phosphatase and osteocalcin (markers of bone formation) and C-telopeptide (marker of bone resorption).

RESULTS

For the analysis of parameters such as number of fractures, bone pain intensity, degree of mobility, weight and height, data were collected from 26 patients, 14 (54%) males and 12 (46%) females; Aged between 2 and 18 years (mean = 9.73 and standard deviation = 5.67), during the period from August 2006 to April 2007 at HINSG in Vitória, ES. Between August 2007 and April 2008, were a analyzed results of bone densitometry and biochemical tests, such as sanguineous C-telopepetide, serum alkaline phosphatase (ALP) osteocalcin from 38 patients, in which 22 (57.9%) were male and 16 (42.1%) female, with ages ranging from 1 to 20 years (mean = 9.44 and standard deviation = 5.47).

Number of Fractures

The data regarding the number of fractures were collected through information with the patient (or parents) or obtained from the medical records. Using these data, the fracture rate by year was calculated and described in Table 1. The statistical analysis of this parameter was performed using Student's t-test. Of the 26 patients analyzed, approximately 77% of them (20/26) had the number of fractures/year decreased during treatment with pamidronate disodium. Among those patients with reduced fracture numbers, 35% (7/20) did not present any fractures after starting the treatment. However, 23% (6/26) had an increasing in the number of fractures/year.

Another analysis can be performed by separating the patients according to the type of OI. In type I patients, the rate of fractures per year before treatment was 1.66, and during treatment this value was reduced to 1.15, a non-statistically significant reduction ($p > 0.05$). However, in type III patients, a significant reduction ($p < 0.05$) was observed between the beginning and during treatment, from 11.1 to 1.58, whereas in type IV patients, a non-significant reduction ($p > 0.05$) was observed, ranging from 5.42 to 1.49.

Pain Intensity Score

Bone pain intensity analysis was performed using an adapted McGrath's visual analogue linear scale. The results of bone pain can be seen in Table 2. Approximately 65% (17/26) of the patients had some degree of reduction in bone pain. About 27% (7/26) of the patients had severe pain before treatment; However, after starting treatment severe pain was no

longer reported. It is also observed that the number of children who do not feel bone pain or have mild pain went from 58% (15/26) to 92% (24/26) after starting treatment.

By analyzing the intensity of bone pain before and during treatment from OI type I patients, we could verify that severe pain varied from 27% (3/11) to 0%, moderate pain from 27% (3/11) to 9% (1/11) and mild pain/painless from 46% (5/11) to 91% (10/11), respectively. All patients from this group had some degree of bone pain before treatment, and during treatment 55% (6/11) of them did not feel any type of pain. In OI type III patients, the severe pain varied from 22% (2/9) to 0%, moderate pain did not change 11% (1/9), and mild pain/painless changed from 67% (6/9) to 89% (8/9). In OI type IV patients, severe pain varied from 33% (2/6) to 0%, mild pain/painless from 67% (4/6) to 100% (6/6) and no patient related moderate pain.

Mobility Degree

The assessment of mobility degree was performed according to the Bleck scale, with 5 points: 0 (bedridden or wheelchair); 1 (walks with support, no leg functionality); 2 (walks indoors, with or without support); 3 (walking for short distances, with or without support) and 4 (walking independently).

The table 3 shows the mobility degree of patients before and during the use of pamidronate disodium. Only in type III OI patients, there was a change in the mobility degree, in which 56% of patients (5/9) uprised 1 degree on the Bleck scale. Only one patient (1/9; 11%) increased 2 degrees. About 33% of patients (3/9) did not change their mobility degrees. In type I OI patients, there was no change in the mobility degree before and during treatment, wherein 82% (9/11) of patients are classified as grade 4 on the Bleck scale, with means they can walk independently. About 18% (2/11) walks for short distances, with or without support (grade 3). In addition, there are no patients bedridden or using a wheelchair. This is because these patients carry the mildest form of the disease, thus they have a smaller number of fractures, less bone deformity, a higher BMD and better bone regeneration. The type IV OI patients also did not show improvement in the mobility degree, as well as seen in type I OI patients. This group of patients shows a variable phenotype and such heterogeneity can be visualized through the analysis of their mobility degrees. About 67% (4/6) of patients can walk independently (grade 4) whereas 33% (2/6) of the patients walk with support, without leg functionality (grade 1).

Prior to treatment, about 33% (3/9) of type III OI patients had a grade 0 (bedridden or wheelchair), 45% (4/9) were classified as grade 1 (walk with support, without leg functionality), and 22% (2/9) as grade 3 (walking for short distances, with or without support). During treatment, there was a reduction up to 22% (2/9) in patients who showed grades 0 or 1. There was also a significant improvement in the mobility of 2 patients (22%) who reached the maximum mobility degree (grade 4). Although there is a reduction in the number of fractures due to treatment with pamidronate disodium, these patients have severe bone deformities and a greater difficulty to ramble independently, due to numerous fractures prior to the use of bisphosphonates (BP).

Weight and Height

The data collected regarding weight and height were obtained during the treatment cycles or through patient charts. With this information curves of age/weight and age/height were constructed and these were compared to the growth curves of children or adolescents without OI (National Center for Health Statistics, 2017, www.cdc.gov/growthcharts) from CDC (Center of Disease Control and Prevention).

All patients had an increase in height during treatment with pamidronate disodium, without significant differences in growth between patients with different types of OI. However, prepubertal type I and III patients had a higher mean growth rate in cm/year compared to type I and III OI patients who are pubescent. In healthy children, the growth rate in prepubertal is about 5-6 cm/year and increases from 8 to 12 cm/year for girls and 10 to 14 cm/year for boys during puberty. In addition, all type III and IV patients are below the 25th percentile in the height parameter when compared to the standard CDC growth curve.

During treatment, approximately 27% (3/11) of type I OI patients with OI type I are below the 5th percentile in the weight parameter according to their ages. By the standard CDC curve, the remaining 73% (8/11) were above the 5th percentile. Only one patient (11.1%; 1/9) with OI type III is within the expected weight for this age, whereas the other 88.9% (8/9) are underweight in relation to their age. However, it can be observed that two patients with OI type III (C2 and C20) had a rapid increase in weight during treatment with pamidronate. In OI type IV patients, 50% (3/6) are below the 5th percentile, while the remainder are above this percentile.

Bone Densitometry

According with the results from the first bone densitometry performed in each patient, at the beginning of the treatment, it was observed that all patients who undergo the treatment had BMC and BMD values below the reference values compared to healthy children. After the start of treatment, an annual bone densitometry was performed to observe the effects of BP therapy in bone metabolism.

About 7.9% (3/38) of the patients followed during this study had only one bone densitometry, thus, it was not possible to compare the skeletal structure modifications of these patients, regarding BMC and BMD. However, 92.1% (35/38) of the patients had two or more densitometries, and the percentage of bone mineral content and bone mineral density per year were calculated (Table 5). In this table, some data are missing because these results could not be verified during the bone densitometry due to the patient's conditions or for technical reasons.

The analysis of bone structure changes was performed according to the OI type. All OI type I patients (18/18) had an average annual increase in bone mineral content (BMC) of the whole body of 33.26% and a standard deviation of 0.16, with a statistically significant increase ($p < 0.05$). Regarding bone mineral density (BMD), two patients (C25/C46; 11.11%) had loss of density.

All patients with OI type III had significant annual increases ($p < 0.05$) in bone mineral density (BMD) and bone mineral content (BMC), both lumbar spine (L1-L4) and the whole body. In OI type IV group, there was an increase in BMC and BMD of the whole body, respectively, 81.56% and 12.62%. However, these increases in bone structure were not statistically significant ($p > 0.10$) probably due to the small number of patients and the great variability of the results. Two OI type IV patients (C11/C56; 20%) did not obtain increases in the bone mass. When analyzed individually, one OI type III (C43) and three OI type IV (C36; C49; C56) had an important increase in the bone mineral content of the whole body. However, one of them (C56) did not show increases in the lumbar spine bone mass.

Regarding the z-score, which represents the measure of the number of standard deviations in relation to the mean expected for the patient's age, two OI type I (C10; C41), two OI type III (C21; C26) and one OI type IV (C11) patients had a reduction in this parameter of the whole body. Among them, three patients (C11; C26; C30) also had a reduction in lumbar spine values. A non-significant ($p > 0.10$) increase in the z-score of patients with OI types I and III can also be observed.

Biochemical Markers

For this study, we evaluated the biochemical markers C-Terminal Cross-Linking Telopeptide (CTX or C-telopeptide), serum alkaline phosphatase (ALP) and osteocalcin. In addition, to exclude other causes of bone fragility such as rickets, primary hyperparathyroidism, Paget's disease and other bone pathologies, the parameters 1,25 dihydroxyvitamin D and 25 hydroxyvitamin D and i-PTH (parathyroid hormone) were also evaluated and all the participants had normal values for these data, thus ensuring the quality of the sample.

The results of 68 tests of the C-Terminal Cross-Linking Telopeptide (CTX) or C-telopeptide parameter revealed that 95% of the exams showed a statistically significant reduction ($p > 0.10$) in the excretion of this peptide after receiving pamidronate disodium for three consecutive days. In situations of increased bone turnover, type I collagen is degraded by osteoclasts, which releases CTX molecules, thus, confirming that the drug acts by inhibiting the action of osteoclasts [4].

Regarding total serum alkaline phosphatase (ALP), 85 exams were performed before and after the infusion of pamidronate disodium in 25 patients with OI, with a reduction of this marker of bone formation in 80% of the tests after the use of the drug. This reduction of total alkaline phosphatase levels throughout the treatment indicates that there was a tendency to balance between formation and bone resorption [5].

As a product of osteoblastic synthesis, osteocalcin (OC) has been used as a marker of bone formation. Circulating osteocalcin was shown to originate primarily from new bone synthesis rather than from the breakdown of bone [6]. This biochemical marker was also evaluated in the sample by 70 tests performed before and after the drug infusion. About 67% of the exams presented an increase in this marker of bone formation. Other results on these biochemical markers can be seen in table 6.

DISCUSSION

Osteogenesis imperfecta is an inherited connective tissue disorder with a large phenotypic and molecular heterogeneity. Due to mutations mostly affecting the collagen type I genes that result in quantitative or qualitative defects, the balance between bone resorption and formation, which normally favors the bone formation, is disrupted in osteogenesis imperfecta. A common issue associated with the molecular abnormality is a disturbance in bone matrix synthesis and homeostasis inducing bone fragility with the removal of thick primary trabeculae and small tissue damages. Bone turnover is higher in children than in adults and still greater in children with osteogenesis imperfecta. Since very early in life, these changes can lead to multiple fractures and progressive bone deformities, including long bone bowing and scoliosis [7].

A multidisciplinary management in osteogenesis imperfecta treatment is required to prevent bone deformities and maximize functional capabilities with significant improvement in life quality. The main benefits of treatment include an improvement in growth, muscle strength, and mobility in patients since birth. The set of actions include physical therapy, medical treatment with bone remodelling drug therapy and orthopaedic surgery, in some cases. Bisphosphonates inhibit the function of osteoclasts and are widely used in the treatment of moderate to severe osteogenesis imperfecta, from infancy to adulthood. Since the cyclic administration of intravenous pamidronate treatment in children with osteogenesis imperfecta resulted in reduction in bone resorption, increase in bone density (BMD), and reduction in bone pain and fracture rate, the use of BP therapy in pediatric patients has been suggested [8].

In addition, recent drug therapies include teriparatide and denosumab [9]. All these therapies target the symptoms and have effects on the mechanical properties of bone due to modification of bone remodelling, therefore influencing skeletal outcome and orthopaedic surgery. Innovative therapies, such as progenitor and mesenchymal stem cell transplantation, targeting the specific altered pathway rather than the symptoms, are being developed [7].

In our study, the cyclic administration of intravenous pamidronate disodium resulted in significant reduction in fracture rate by year in approximately 77% (20/26) of the sample. Among those patients with reduced fracture numbers, 35% (7/20) did not present any fractures after starting the treatment. However, 23% (6/26) had a slightly increasing in the number of fractures/year. Castillo and colleagues [10] verified a reduction in non-vertebral fracture rate in several studies and was statistically significant in three studies analyzed. The reduction in fracture rate was clinically significant, ranging from 30 to 60%.

The most benefited were the OI type III patients, with 89% of individuals presenting reduction in fracture rate, with a significant reduction ($p < 0.05$) was observed between the beginning and during treatment, from 11.1 to 1.58 (Table 1). In type I patients group, the rate of fractures per year before treatment was 1.66, and during treatment this value was reduced to 1.15, a non-statistically significant reduction ($p > 0.05$), whereas in type IV patients, a non-significant reduction ($p > 0.05$) was observed, ranging from 5.42 to 1.49. However, the small reduction or even an increase in the number of fractures in almost 47% of all OI type I/IV patients together, in some cases, may be due to small increases in BMD and improvement in mobility

and physical activity due to the decrease of bone pain, which means a significant improvement in life quality for these patients.

A clinical study comprising severely affected osteogenesis imperfecta children under 3 years of age for a period of 12 months (intravenous pamidronate in 3 consecutive days for four to eight cycles) resulted in an increased BMD in treated children up to 227% ($p < 0.001$), and a significant decreased in fracture rate ($p < 0.01$) compared to untreated control children group [11]. Thus, confirming that intravenous pamidronate administration in children under 3 years of age and severely affected osteogenesis imperfecta children is beneficial.

In another study, intravenous pamidronate for 1 year to types III and IV osteogenesis imperfecta children decreased the fracture rates only in upper extremities, and functional mobility and pain were not improved. Even extending therapy for 1 year did not additionally significantly improve the bone density. However, there was a significant increased lumbar spine BMD Z-scores and volumetric vertebral size [12].

Although BMD increases have been reported during these treatments, fracture data are still inconclusive [13]. Especially because any improvements in mobility resulted from an improved clinical status may lead to a higher risk of fractures. In some cases, the proportion of patients who experienced a fracture was not significantly reduced by BP therapy. Long-bone fracture rates can still occur as well as a progressive scoliosis [9, 14].

Bisphosphonates are currently prescribed to children and adults with osteogenesis imperfecta. It is expected that oral or intravenous BP increase bone mineral density in this condition. Given their current widespread and expected continued use, the optimal method, duration of therapy and long-term safety of BP therapy require further investigation. In addition, long-term fracture and bone pain intensity reductions allow a better life quality for patients. But, the possibility of a placebo effect regarding the improvement of bone pain can not be ruled out. Although many clinical trials in pediatric patients reported significant gains in BMD and decrease in pain compared to placebo with BP, they did not conclude that BP significantly reduce the incidence of fractures in osteogenesis imperfecta [8]. When we analyze the data regarding the number of fractures, we cannot discard the memory bias that was present during the data collection, in some cases, because of lacking the exactly number of fractures suffered before the treatment.

Bone pain intensity analysis was performed using an adapted McGrath's visual analogue linear scale. Once it is a subjective parameter with different thresholds of pain sensitivity, it is hard to estimate the intensity, as they might have a certain amount of bone pain due to multiple fractures since perinatal period, during childhood in the developing skeleton and bone compressions, for example. Following this way, patients tend to complain only when they suffer extensive fractures. Bone pain may be reported by patients who often undergo multiple surgical procedures, reduced mobility and ligamentous laxity.

The use of BP in relieving bone pain has been reported for decades in many areas, including oncological and orthopedic diseases [15, 16]. Glourieux and colleagues [16] showed that the use of this class of drugs allows an early and marked reduction of chronic bone pain in patients with OI. Pain reduction allows more effectiveness in physical therapy, a positive result in mobility that can also affect the fractures rate [17].

As reported by Seikaly and colleagues [18], we also demonstrated the reduction in pain and the analgesic use of BP in bone pain relief. In our sample (n=26), all patients had some reduction in bone pain degree after treatment. In OI type I patients, we verify that severe pain varied from 27% (3/11) to 0%, moderate pain from 27% (3/11) to 9% (1/11) and mild pain/painless from 46% (5/11) to 91% (10/11), respectively. All patients from this group had some degree of bone pain before treatment, and during treatment 55% (6/11) of them did not feel any type of pain. In OI type III patients, the severe pain varied from 22% (2/9) to 0%, moderate pain did not change 11% (1/9), and mild pain/painless changed from 67% (6/9) to 89% (8/9). In OI type IV patients, severe pain varied from 33% (2/6) to 0%, mild pain/painless from 67% (4/6) to 100% (6/6) and no patient related moderate pain.

The most remarkable reduction was in those patients who related severe pain before treatment. Moreover, the painless group increased from 23% to 61%, which demonstrates a migration from other categories of pain scale. It is important to notice that a positive effect of cyclic BP therapy in children with OI is the reduction in bone pain immediately following treatment, which is sustained for several weeks until the next infusion. However, additional studies should be performed to evaluate the etiology of bone pain reduction.

Skeletal deformities in lower limbs due to viciously consolidated fractures are directly related to the severity of the fracture and interfere in prognosis of mobility degree for each patient. Some patients with osteogenesis have severe limb deformities due to fractures occurring before the start of treatment and thus, the reduction in the number of fractures may not improve the degree of mobility. However, if treatment with BP starts early (before deforming fractures occur) its clinical effects and life quality can be significantly improved. Moderate and severe forms of OI are associated with greater functional limitation due to fracture history but show a significant improvement in their ability to participate in high level activities after one year of pamidronate treatment [19].

As demonstrated in table 3, the degree of mobility is quite variable among the different types of OI. For example, type I patients, milder form of the disease, do not have bone deformity, thus, they can walk independently or with small restrictions. Type III carriers, due to the countless fractures, have severe bone deformities, having their mobility affected immensely. In general, these patients are bedridden, in a wheelchair or walk without the functionality of the legs (eg, dragging themselves seated). Type IV patients, however, have variable ambulatory characteristics, in which patients can walk independently while others are confined to the wheelchair.

For a better insight of pamidronate effects on growth, it would be appropriate to compare the weight and height of treated and untreated OI patients. However, the growth of pamidronate treated patients compared to healthy children for ethical reasons. As a characteristic of the disease, the baseline height is commonly low for age in all OI types [2].

In our study, all OI types III and IV patients who were under pamidronate treatment are still below the 25th percentile in the height parameter, when compared to children and adolescents without the disease. The short stature may come as the result of vertebral compression fractures, scoliosis, lower limb deformity, or disintegration of the epiphyses. However, growth may be slow also in the absence of these abnormalities. The etiology of this growth restriction is not known. We suggest being a protective mechanism since any mechanical load creates less stress in short than long bones so

that, short bones would break less easily. However, the use of pamidronate may increase growth rates preventing deformities in long bones and vertebral collapses by BMD increasing.

Pre-puberty children had a higher growth rate in height when compared to puberty children possibly because in with OI patients the growth rate is greatly reduced at 6 or 7 years, and often stops a bit later. Thus, the OI patients from our study group who are at puberty probably started treatment at more advanced ages, so they already had a series of bone metabolism alterations, which would act decreasing a beneficial effect of pamidronate in growth rate. There was a weight increasing as expected for their height changes. This study, as well as others already published, shows that some patients had a rapid weight gain during treatment with pamidronate, and this is of interest since excess weight puts additional loads on the skeleton and interferes with rehabilitation. However, it has not yet been clarified how pamidronate influences this weight gain [2].

Since the last decades, several studies have reported the beneficial effects of long-term intravenous pamidronate in OI [2, 17, 20]. Zeitlin and colleagues [2] reported that after 1 year of pamidronate therapy, height z scores had increased significantly in type III OI (by 0.3 ± 0.8, mean ± standard deviation; P = .04) and did not change in types I and IV. Weight z scores increased significantly in type I OI (by 0.2 ± 0.4, P = .01). After 4 years of pamidronate therapy, mean height z scores increased significantly in type IV OI (by 0.41 ± 0.71, P = .04), whereas nonsignificant increasing were found for type I and III. Long-term pamidronate therapy was associated with a significant height gain in all three OI types (P < .001). For some patients, who reached final height after 3.0 ± 1.0 years of treatment, they were taller on average than expected for untreated patients (P = .04).

Regarding the z-score, which represents the measure of the number of standard deviations in relation to the mean expected for the patient's age, two OI type I (C10; C41), two OI type III (C21; C26) and one OI type IV (C11) patients had a reduction in this parameter of the whole body. Among them, three patients (C11; C26; C30) also had a reduction in lumbar spine values. These results may be due to multifactorial elements that affect the response to BP treatment, e.g. age, puberty degree, bone maturity, body mass index, nutritional status and OI type [21, 22]. A non-significant ($p > 0.10$) increase in the z-score of patients with OI types I and III can also be observed.

The z-score measurements, when calculated based on a population with normal weight, height and bone density, will not be an accurate indicator to assess patients with OI. However, if the z-score is corrected for the study population, this parameter is excellent for assessing the effects of pamidronate therapy [17]. It is important to emphasize the difficulty of performing bone densitometry in patients with OI, especially regarding bone mineral density (BMD), due to the presence of metallic instrumentation, difficulties in positioning the patient, movements and presence of fractures. In addition, very low values of BMD may sometimes result in poorly accurate values [21].

The response to pamidronate treatment may vary from each patient. Some patients have greater gains in bone mass than others. This is due to the individual response to the drug that is influenced by several factors such as age, Tanner stage, bone maturity, body mass index, nutritional status and type of OI [21]. The main increases in bone mass, with a consequent increase in BMD, BMC and z-score, occur during growth. This increase in bone mass is the main goal of treatment with

pamidronate in patients with OI, because, consequently, there is a reduction in the number of fractures, improved mobility, bone pain and quality of life of patients.

Patients undergoing treatment for three years or more may present stability or even decrease in their bone mass increments [20]. In some patients, even with an increasing in bone density, some fractures continue to occur. This fact can be explained in three ways. First, despite the increase in bone density, it remains well below the normal limit. Second, the bone fragility in OI can remain despite the increase or even normality of the density. Third: patients with OI who receive the treatment feel better and increase the level of daily activities, with greater exposure to the risk of fractures [21]. Early treatment at early ages prevented and even altered the course of bone deformity occurring in patients with OI, especially deformity of the vertebrae [17].

Currently, assays for bone formation include the N-terminal propeptide of type I collagen, bone-specific alkaline phosphatase, and intact or N-mid osteocalcin in serum. Resorption assays include collagen N-telopeptide, C-telopeptide, tartrate-resistant acid phosphatase and urinary mid-molecule osteocalcin [23]. All have a circadian variation and respond to changes in bone formation and resorption accompanying growth, age, menopause, metabolic bone diseases and medications that affect bone turnover [6].

Both the formation markers (alkaline phosphatase and osteocalcin) and the reabsorption markers (C-telopeptide) used in this study were measured in the serum, being this type of collection more convenient and not requiring additional creatinine measurements to correct the values obtained. However, it should be emphasized the importance of care when collecting serum samples for these markers, since they have a circadian rhythm of excretion. In addition, changes in renal function may significantly interfere with the metabolism and excretion of biochemical markers, especially osteocalcin. This marker of bone formation also undergoes menstrual cycle and genetic influences [24].

The reduction of C-telopeptide and alkaline phosphatase markers indicate that bone turnover decreased during therapy due to changes in the balance between formation and resorption that favored the increase of bone mass. The decrease in the systemic activity of bone resorption, represented by the reduction of C-telopeptide levels, are mainly responsible for the therapeutic effects of BP in osteopenia-like diseases such as Osteogenesis Imperfecta. However, the process of bone turnover enables bones to repair microscopic damage and maintain their strength and integrity [25]. In addition, the reduction of alkaline phosphatase in the first three days after the start of the therapy cycle is not due to a change in the activity of bone remodeling, since this process would take a few weeks to occur, and the direct effect of pamidronate on one or both types of skeletal cells expressing this enzyme, osteoblasts and chondrocytes can be the most plausible explanation.

CONCLUSION

Our data support the effectiveness in reduction of bone pain after intravenous pamidronate and the significant reduction in fracture rates in patients with OI type III, showing that this group are the most benefited with this treatment. In addition, we suggest that the treatment allows the growth and mobility of patients with OI. Because of heterogeneity and

small size of the OI type IV sample, we cannot make any inference from this group about the results obtained. The OI type I patients showed a significant improvement in the decrease of bone pain and a slight but not significant improvement in other parameters analyzed. Due to these results, the idea that pamidronate should be used in the treatment of all forms of OI was reinforced. An increase in bone mass after intravenous pamidronate infusion, with consequent decrease in fracture rates, improvement of bone pain and patient mobility is expected. The decrease in C-telopeptide and serum alkaline phosphatase values presented in our results confirm the reduction of bone turnover after starting treatment. As well as the levels of serum concentrations of total osteocalcin are established measurement for bone formation. In conclusion, long-term intravenous BP therapy was associated with higher Z-scores for lumbar spine and bone mineral density.

DISCLOSURE STATEMENT

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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AUTHOR'S CONTRIBUTIONS

The principal researchers of the work were Marcos Vinícius Dornelas de Moraes, Miquele Milanez and Flavia de Paula. Miquele Milanez obtained clinical data of the participants. Maria Regina Galveas Oliveira Rebouças, Akel Nicolau Akel Jr, Valentim Sipolatti and Vanda Regina Rangel Nunes helped with the clinical assessment of the patients. Marcos Vinícius Dornelas de Moraes, Miquele Milanez and Flavia de Paula wrote the manuscript. Flavia Imbroisi Valle Errera helped with suggests for the manuscript discussion. All authors review the manuscript.

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Table 1 - Clinical characteristics and fractures number of OI patients treated with disodium pamidronate

PATIENTS (n=26)	OI TYPE	AGE	SEX	NUMBER OF FRACTURES	
				BEFORE TREATMENT	AFTER TREATMENT
C5	I	13	M	0,27	0
C6	I	16	M	0,14	2
C7	I	11	M	1,14	1,15
C9	I	14	F	0,21	0,66
C10	I	18	M	0,98	0
C25	I	6	F	2,6	2,66
C27	I	5	M	7,06	3,33
C31	I	18	M	0,11	0
C37	I	3	F	4,76	1,5
C39	I	2	M	0	1,42
C42	I	12	F	1,02	0
Mean		**1,73**	**2,3M:1F**	**1,66**	**1,15**
Standard Deviation		**5,85**		**2,29**	**1,16**
C2	III	16	M	1,06	1,17
C12	III	9	M	21,25	2,62
C20	III	15	M	9,37	1,5
C21	III	18	F	5,8	0,88
C22	III	19	F	5,8	0
C24	III	3	M	2,72	1,42
C26	III	5	M	22,7	0,32
C33	III	5	F	14,6	3,88
C45	III	7	F	16,6	2,44
Mean		**10,77**	**1,2M:1F**	**11,1**	**1,58**
Standard Deviation		**6,22**		**7,99**	**1,22**
C8	IV	11	M	11,24	1,2
C11	IV	10	M	0,28	0
C23	IV	4	F	2,2	0
C30	IV	5	F	4,5	2,34
C36	IV	2	F	14	3
C41	IV	6	F	0,31	2,38
Mean		**6,33**	**1M:2F**	**5,42**	**1,49**
Standard Deviation		**3,5**		**5,85**	**1,29**

Table 2 - Bone pain evaluation before and during patient's treatment acording to the adapted McGrath's visual analogue linear scale

GROUPS (n=26)	BONE PAIN							
	BEFORE TREATMENT				DURING TREATMENT			
	No Pain	Mild Pain	Moderate Pain	Severe Pain	No Pain	Mild Pain	Moderate Pain	Severe Pain
All Patients	*23%*	*35%*	*15%*	*27%*	*61%*	*31%*	*8%*	*0%*
OI Type I	*0%*	*46%*	*27%*	*27%*	*55%*	*36%*	*9%*	*0%*
OI Type III	*34%*	*33%*	*11%*	*22%*	*67%*	*22%*	*11%*	*0%*
OI Type IV	*50%*	*17%*	*0%*	*33%*	*67%*	*33%*	*0%*	*0%*

Table 3 - Degree of mobility evaluation in patient with Osteogenesis Imperfecta before and during treatment

GROUPS (n=26)	DEGREE OF MOBILITY									
	BEFORE TREATMENT					DURING TREATMENT				
	0	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
OI Type I	*0%*	*0%*	*0%*	*18%*	*82%*	*0%*	*0%*	*0%*	*18%*	*82%*
OI Type III	*33%*	*45%*	*0%*	*22%*	*0%*	*23%*	*22%*	*22%*	*11%*	*22%*
OI Type IV	*0%*	*33%*	*0%*	*0%*	*67%*	*0%*	*33%*	*0%*	*0%*	*67%*

Table 4 - Effects of pamidronate on weight and height

OI Type I (Prepubescent)	**sex**	**cm**	**year**	**cm/year**	**weight/year**
C27	M	22,5	3	7,5	2,26
C7	M	13,5	2,25	6	4,92
C10	M	13,5	2	6,75	2,1
C39	M	19	1,75	10,8	2,48
C25	F	12	2,58	4,65	1,82
C37	F	23,5	2,08	11,29	2,76
C27	M	22,5	3	7,83	2,72
Mean				**2,67**	**1,12**
Standard Deviation				**7,5**	**2,26**
OI Type I (Pubescent)	**sex**	**cm**	**year**	**cm/year**	**weight/year**
C5	M	14	2	7	5,29
C6	M	7	1,83	3,82	2,1
C31	M	10	1,33	7,52	2,56
C9	F	22	3,83	5,74	4
C42	F	20	4	5	2,5
Mean				**5,82**	**3,29**
Standard Deviation				**1,5**	**1,33**
OI Type III (Prepubescent)	**sex**	**cm**	**year**	**cm/year**	**weight/year**
C12	M	23,5	4,08	5,76	1,46
C24	M	16	1,42	11,27	0,84
C26	M	26	3,75	6,93	1,25
C33	F	32	3,67	8,72	2,37
C45	F	7	1,67	4,19	1,47
Mean				**7,37**	**1,48**
Standard Deviation				**2,74**	**0,56**
OI Type III (Pubescent)	**sex**	**cm**	**year**	**cm/year**	**weight/year**
C2	M	12,7	2,33	5,45	6,14
C20	M	15	2,5	6	5,6
C21	F	4	4,5	0,89	0,13
C22	F	2	3	0,67	1,42
Mean				**3,25**	**3,32**
Standard Deviation				**2,87**	**3**
OI Type IV (Prepubescent)	**sex**	**cm**	**year**	**cm/year**	**weight/year**
C8	M	14	3	4,67	4,11
C11	M	4	0,67	5,97	2,18
C23	F	12	2,25	5,33	3,52
C30	F	17	3,67	4,63	1,01
C36	F	9,5	1	9,5	2,55
C41	F	1	0,41	2,44	0
Mean				**5,42**	**2,23**
Standard Deviation				**2,32**	**1,53**

Table 5 - Clinical features and changes observed in bone densitometry of patients with OI treated with pamidronate disodium

PATIENT (n=38)	SEX	AGE	OI TYPE	BMC		BMD		Z-score	
				L1-L4	Whole Body	L1-L4	Whole Body	L1-L4	Whole Body
C46	F	5	I	27,46%	19,93%	18,93%	-5,75%	**	**
C27	M	6	I	14,51%	53,35%	3,07%	8,27%	**	**
C47	M	8	I	35,77%	30,07%	20,33%	5,61%	0,589	0,28
C7	M	12	I	52,75%	17,27%	19,11%	4,96%	0,26	0,21
C41	F	8	I	**	27,59%	**	1,20%	**	-0,37
C5	M	15	I	**	25,02%	15,45%	2,32%	**	**
C9	F	15	I	33,52%	23,71%	21,25%	7,95%	0,78	0,31
C50	M	7	I	28,93%	34,63%	18,96%	3,30%	**	**
C42	F	13	I	**	12,02%	**	4,08%	**	**
C6	M	18	I	**	50,51%	21,99%	3,09%	**	**
C51	M	3	I	**	53,66%	**	11,45%	**	**
C25	F	8	I	106,92%	51,51%	53,44%	-2,21%	1,66	0,8
C37	F	4	I	**	33,55%	22,58%	11,68%	**	**
C31	M	19	I	24,41%	19,47%	14,12%	7,59%	0,06	0,48
C10	M	10	I	27,36%	25,64%	11,90%	4,80%	0,21	-0,03
C39	M	4	I	**	68,06%	**	16,41%	**	**
C55	M	19	I	**	19,44%	17,08%	8,27%	**	**
C53	F	9	I	*	*	*	*	*	*
Mean	**1,6M:1F**	**10,17**		**39,07%**	**33,26%**	**19,86%**	**5,47%**	**0,59**	**0,24**
STD		**5,27**		**0,27**	**0,16**	**0,11**	**0,05**	**0,58**	**0,37**
C20	M	16	III	116,36%	29,51%	43,75%	0,27%	0,51	0,26
C43	F	2	III	**	624,06%	**	36,56%	**	**
C33	F	6	III	44,95%	26,12%	10,54%	4,65%	**	**
C26	M	7	III	31,15%	54,84%	27,15%	4,93%	-0,28	-0,14
C45	F	8	III	43,65%	16,75%	12,37%	4,82%	0,71	0,36
C52	M	12	III	*	*	*	*	*	*
C2	M	17	III	18,50%	39,56%	35,84%	9,37%	1,01	0,41
C22	F	20	III	23,05%	20,61%	43,23%	4,14%	0,88	0,05
C21	F	19	III	23,08%	22,49%	14,21%	5,46%	0,42	-0,07
C12	M	10	III	17,62%	23,27%	7,04%	4,41%	0,21	0,23
Mean	**1M:1F**	**11,7**		**39,80%**	**95,25%**	**24,27%**	**8,29%**	**0,49**	**0,15**
STD		**6,09**		**0,32**	**1,98**	**0,15**	**0,1**	**0,43**	**0,21**
C8	M	12	IV	32,80%	17,74%	4,86%	2,56%	0,126	0,14
C49	F	8	IV	**	111,12%	**	57,49%	**	0,55
C24	M	5	IV	**	37,73%	**	5,19%	**	**
C36	F	3	IV	**	146,48%	**	8,95%	**	**
C30	F	7	IV	17,54%	11,36%	3,46%	2,26%	-0,52	0,1
C23	F	5	IV	49,95%	35,51%	17,81%	5,87%	**	**
C11	M	11	IV	-8,97%	-3,55%	-7,65%	-2,66%	-0,69	-0,39
C56	M	3	IV	-21,52%	348,45%	-24,42%	26,72%	**	**
C54	M	4	IV	36,21%	29,16%	20,57%	7,21%	**	**
C48	M	1	IV	*	*	*	*	*	*
Mean	**1,5M:1F**	**5,9**		**17,67%**	**81,56%**	**2,44%**	**12,62%**	**-0,36**	**0,1**
STD		**3,57**		**0,27**	**1,11**	**0,16**	**0,18**	**0,43**	**0,38**

* Patients who have only one bone densitometry were not analysed due to impossibility to calculate annual rates of change of BMC, BMD and z-score. ** Values not provided in bone densitometry results.

Table 6 - Biochemical markers c-telopeptide, serum alkaline phosphatase and osteocalcin, before and after pamidronate infusion

BIOCHEMICAL MARKERS	C-telopeptide (n = 68)		Serum Alkaline Phosphatase (n = 85)		Osteocalcin (n = 70)	
	Before	**After**	**Before**	**After**	**Before**	**After**
Mean	0,712	0,297	270,93	239,86	62,76	72,66
STD	0,397	0,129	90,56	84,63	39,34	49,53

*Before: Indicates that the tests were collected on the first day of the cycle, prior to the pamidronate infusion. **After: Indicates that the tests were collected on day 3 of the cycle after pamidronate infusion.

5 CONCLUSÃO

Por meio da análise dos genes COL1A1, COL1A2, P3H1 e FKBP10 foi possível identificar aproximadamente 95% das mutações causadoras da Osteogênese Imperfeita na amostra estudada. Em adição, nossos dados indicam uma eficácia na redução da dor óssea após administração do pamidronato intravenoso e a redução significativa nas taxas de fratura em pacientes com OI tipo III, mostrando que esse grupo é o mais beneficiado com este tratamento. Devido à heterogeneidade e ao tamanho pequeno da amostra OI tipo IV, não podemos fazer nenhuma inferência deste grupo sobre os resultados obtidos. Os pacientes com OI tipo I mostraram uma melhora significativa na diminuição da dor óssea e uma leve mas não significativa melhora em outros parâmetros analisados. A diminuição dos valores de C-telopeptídeo e fosfatase alcalina sérica apresentados confirmam a redução do turnover ósseo após o início do tratamento. Os níveis de concentração sérica de osteocalcina total observados são parâmetros indicativos de formação óssea. Com base nestes resultados, sugere-se que o pamidronato deve ser utilizado no tratamento de todas as formas de OI.

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