UNIVERSIDADE FEDERAL DO CEARÁ
FACULDADE DE FARMÁCIA, ODONTOLOGIA E ENFERMAGEM
PROGRAMA DE PÓS GRADUAÇÃO EM ODONTOLOGIA

THYCIANA RODRIGUES RIBEIRO

RAQUITISMO HIPOFOSFATÊMICO FAMILIAR – ESTUDO SOBRE PEPTÍDEOS SALIVARES E ESTRUTURA MINERAL DENTÁRIA

FORTALEZA
2013

THYCIANA RODRIGUES RIBEIRO

RAQUITISMO HIPOFOSFATÊMICO FAMILIAR – ESTUDO SOBRE PEPTÍDEOS SALIVARES E ESTRUTURA MINERAL DENTÁRIA

Tese submetida à Coordenação do Curso de Pós-Graduação em Odontologia da Universidade Federal do Ceará, como requisito parcial para obtenção do grau de Doutora em Odontologia.

Área de Concentração: Clínica Odontológica

Orientadora: Profa. Dra. Cristiane Sá Roriz Fonteles

Fortaleza
2013

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca de Ciências da Saúde

R372r Ribeiro, Thyciana Rodrigues.
Raquitismo hipofosfatêmico familiar: estudo sobre peptídeos salivares e estrutura mineral dentária/ Thyciana Rodrigues Ribeiro. – 2013.
111 f. : il.

Tese (Doutorado) - Universidade Federal do Ceará. Faculdade de Farmácia, Odontologia e Enfermagem. Programa de Pós-Graduação em Odontologia, Fortaleza, 2013.
Área de Concentração: Clinica Odontológica.
Orientação: Prof. Dra. Cristiane Sá Roriz Fonteles.

1. Raquitismo Hipofosfatêmico Dominante Ligado ao Cromossomo X. 2. Microtomografia por Raio-X. 3. Peptídeos. 4. Saliva. 5. Cromatografia Líquida de Alta Pressão. 6. Proteínas. 7. Eletroforese. I. Título.

CDD 616.395

THYCIANA RODRIGUES RIBEIRO

RAQUITISMO HIPOFOSFATÊMICO FAMILIAR – ESTUDO SOBRE PEPTÍDEOS SALIVARES E ESTRUTURA MINERAL DENTÁRIA

Tese apresentada à Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará como requisito parcial para obtenção do grau de Doutora em Odontologia

Aprovada em:___/___/___

BANCA EXAMINADORA

_____

Profª. Dra. Cristiane Sá Roriz Fonteles (Orientadora)
Universidade Federal do Ceará

_____

Prof. Dr. Eduardo Costa Studart Soares
Universidade Federal do Ceará

_____

Prof. Dr. Fabrício Bitu Sousa
Universidade Federal do Ceará

_____

Profª. Dra. Cláudia Ferreira Santos
Universidade Estadual do Ceará

_____

Prof. Dr. Nilberto Robson Falcão do Nascimento
Universidade Estadual do Ceará

Dedico este trabalho em primeiro lugar a Deus,
a minha mãe Lúcia e ao meu pai Silvio.

AGRADECIMENTOS

Agradeço em primeiro lugar a Deus pela minha vida, por tudo o que tenho e sou; pela minha força de vontade e determinação, elementos essenciais na minha caminhada.

Ao meu pai Silvio e a minha mãe Lúcia, exemplos de dignidade e honestidade, por todo amor, carinho e união da nossa família, pilares da minha educação e formação moral. Sem vocês tenho certeza que não chegaria até aqui. Muito obrigada por serem as duas pessoas mais importantes e presentes da minha vida.

A toda a minha família que se fez presente em algum momento da minha vida, certamente trazendo alguma contribuição, principalmente a minha tia Silvia Fernandes, que desde o início foi uma grande incentivadora para a minha carreira acadêmica.

Ao meu namorado Fábio Wildson Gurgel Costa, uma pessoal especial, eterno incentivador para todas as minhas conquistas, sempre atento as minhas dúvidas e inquietações e companheiro em todos os momentos com perseverança sempre. Obrigada pelo auxílio na viabilização dos pacientes, pelo seu carinho, cuidado e amor incondicionais.

A minha orientadora e grande amiga, professora Dra. Cristiane Sá Roriz Fonteles, por esses 10 anos de convivência e ensinamentos substanciais ao meu crescimento científico, profissional e pessoal. Pelo exemplo de força, caráter, determinação e superação, e por ser presente, viabilizando sempre o melhor, independente do lugar e das condições climáticas. Muito obrigada por tudo.

Ao Dr. James Williams, pela consideração e acolhimento em Indianápolis, essenciais no primeiro ano desse doutorado. Por ter me recebido em seu laboratório para que as análises de micro-CT fossem realizadas. A sua família e à Molly pela atenção proporcionada.

À professora Dra. Cláudia Ferreira Santos, por toda a paciência, ensinamentos, ajuda e facilidade de acesso ao Laboratório da Universidade Estadual do Ceará, durante a etapa de análise das proteínas, e por ter aceitado o convite para compor a banca avaliadora dessa tese.

Ao professor Dr. Manassés Claudino Fonteles, pela importante contribuição e orientação durante a análise dos peptídeos, através do HPLC, por ter me recebido no laboratório de Farmacologia Metabólica e Fisiologia Celular / Plaquetas, sob sua coordenação na Universidade Federal do Ceará para que essa etapa pudesse ser realizada e, também, por aceitar o convite para fazer parte da banca avaliadora da presente tese.

Ao professor Dr. Fábio Sampaio, da Universidade Federal de João Pessoa, pela atenção em fornecer os coletores de saliva, “Lashley cups”.

Ao professor Dr. Eduardo Studart, por ter viabilizado o acesso aos pacientes com raquitismo, do presente estudo, e por ter aceitado ao convite de compor a banca da presente tese.

Ao professor Dr. Fabrício Bitu, por ter aceitado o convite para compor a banca do presente trabalho. Ao professor Nilberto, por ter aceitado o convite para suplente da banca de defesa da presente tese.

Aos professores, Dr. Henrique Clasen Scarparo e Dr. Tácio Pinheiro Bezerra, por terem feito importantes considerações durante a pré-defesa dessa tese. Não esquecendo também de agradecer por mais essa contribuição ao Dr. Fábio Wildson.

À professora Dra. Lidiany Karla Azevedo Rodrigues, coordenadora do Programa de Pós-Graduação em Odontologia da UFC, pelo apoio e atenção sempre prestados.

Ao professor Sérgio Lima Santiago, que não cessou esforços para o apoio prestado durante o período em que foi coordenador do Programa de Pós Graduação em Odontologia.

A todos os professores do Programa de Pós Graduação em Odontologia da Universidade Federal do Ceará.

Às funcionárias do Programa de Pós Graduação em Odontologia, Lúcia e Janaíne pela ajuda e atenção. Às bibliotecárias Rosa e Maria Aires, da Universidade Federal do Ceará, pela atenção com as padronizações bibliográficas. À funcionária Silvia pelo auxílio prestado.

A todos os colegas de doutorado pela convivência e momentos compartilhados. Aos meus amigos de grupo de pesquisa Karla Shangela (amiga, ajudante e estatística!), Juliana Ximenes, Rebeca, Luciano, Érico e Beatriz, pelo companheirismo do nosso grupo de pesquisa e pela amizade.

Aos colegas da graduação, Johnatan, César e Hilana, que auxiliaram com empenho e dedicação os procedimentos realizados durante a etapa do HPLC.

A todos os meus amigos e colegas que contribuíram com meu desenvolvimento pessoal.

À professora Marivan Ferraro pela realização da revisão gramatical.

À CAPES pelo apoio financeiro e manutenção da bolsa de auxílio.

Àqueles que injustamente não foram citados, mas que certamente contribuíram muito para a realização desse trabalho.

“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas graças a Deus, não sou o que era antes.”

Marthin Luther King

RESUMO

Raquitismo hipofosfatêmico ligado ao cromossomo X (XLHR) é a maior causa de raquitismo hereditário, com uma incidência de 1:20.000 nascidos vivos, representando mais de 80% das formas de raquitismo hipofosfatêmico familiar. A saliva é o fluido humano mais disponível e de fácil acesso, o que faz dela uma das ferramentas mais pesquisadas no diagnóstico de patologias. Nesse contexto, essa tese, constituída de 4 artigos objetivou: (1) descrever as principais manifestações sistêmicas, achados orais e tratamentos dentários em 3 gerações de uma família afetada; (2) analisar o padrão de mineralização do esmalte e da dentina nos pacientes afetados por XLHR, utilizando microtomografia computadorizada (Micro CT), e associar a mineralização do esmalte e da dentina em dentes decíduos e permanentes, segundo gênero e presença/ausência da doença; (3) avaliar o perfil de peptídeos na saliva de pacientes com XLHR, utilizando cromatografia líquida de alta performance (HPLC); e (4) caracterizar proteínas salivares nessa condição, utilizando eletroforese unidimensional. No estudo 1, exames orais, laboratoriais e avaliações histológicas, tomografias computadorizadas cone-beam e radiografias periapicais foram realizadas para a apropriada instituição da estratégia de tratamento mais adequada. No estudo 2, dentes foram coletados de 5 indivíduos de uma mesma família. Gênero, idade, posição dentária (anterior/posterior) e tipo dentário (decíduo/permanente) foram registrados para cada paciente. Após a coleta, os dentes foram colocados em solução de timol a 0,1% até a análise através do Micro CT. As imagens projetadas foram reconstruídas e analisadas. No estudo 3, saliva total não estimulada e saliva de parótida estimulada foram obtidas de 8 indivíduos afetados com (AFF) e 8 indivíduos sem (CON) XLHR, de ambos os gêneros e idades entre 8 e 66 anos. Sobrenadantes foram analisados por meio de HPLC e o fluxo salivar (mL/min) foi calculado. Os picos que se apresentaram maiores nos cromatogramas do HPLC foram caracterizados. No estudo 4, saliva total não estimulada e saliva de parótida estimulada também foram obtidas, sendo a concentração de proteínas totais determinada pelo Método do Ácido Bicinconínico (BCA). Proteínas foram caracterizadas de acordo com o peso molecular através de eletroforese unidimensional. O estudo 1 mostrou a importância do conhecimento dos sinais e sintomas clínicos do XLHR para o correto diagnóstico dessa doença, e para o estabelecimento de atendimento odontológico preventivo e abrangente. No artigo 2, os dentes de todos os pacientes afetados apresentaram dentina com padrão de mineralização diferente comparado aos dentes de indivíduos saudáveis, sendo os defeitos na dentina observados próximo às câmaras pulpares. No artigo 3, os fluxos salivares da saliva total e de parótida foram significativamente diferentes ($p=0,001$), sendo o fluxo de saliva total maior ($0,518 \pm 0,282$ mL/min) do que o de saliva de parótida ($0,124 \pm 0,086$ mL/min). O fluxo salivar da saliva total foi maior no grupo AFF ($0,698 \pm 0,229$) que no grupo CON ($0,339 \pm 0,210$ mL/min) ($p = 0,006$). Vinte e oito picos foram encontrados em saliva total e 21 em saliva de parótida. A saliva total do grupo CON apresentou menor número de picos que a do grupo AFF. Na saliva de parótida, os picos 17 e 28 (tempos de retenção: 24 e 39 min) foram encontrados exclusivamente no grupo AFF e o pico 13 (tempo de retenção: 19 min) no CON. Artigo 4 demonstrou diferença relacionada à concentração de proteínas totais entre saliva total e de parótida ($p < 0,001$), sendo a maior concentração encontrada na saliva total ($102,603 \pm 42,336$ µg/mL) que na saliva de parótida ($0,699 \pm 0,438$ µg/mL). Bandas com 102 kDa, 48 kDa e 24 kDa apresentaram maior intensidade na saliva total do grupo CON ($p = 0,015$, $p = 0,043$ e $p = 0,022$). Em conclusão, pacientes com XLHR apresentaram características específicas relacionadas à mineralização dentinária e proteínas e peptídeos salivares que podem levar à diferenciação desses pacientes de indivíduos saudáveis, avançando no campo diagnóstico.

Palavras chave: Raquitismo hipofosfatêmico ligado ao cromossomo X, Microtomografia computadorizada, Peptídeos, Saliva, HPLC, proteínas, eletroforese.

ABSTRACT

X-linked hypophosphatemic rickets (XLHR) is the most common cause of heritable rickets, with an incidence of 1:20,000 live births, representing more than 80% of familial hypophosphatemic rickets. Saliva is the most easily available and accessible body fluid, which makes it one of the most sought after tools in diagnostic pathology. In this context, this thesis, constituted by 4 articles aimed to: (1) describe the main systemic manifestations, oral findings and dental management in 3 generations of an affected family; (2) analyze the mineralization pattern of enamel and dentin in patients affected by XLHR using micro-CT, and to associate enamel and dentin mineralization in primary and permanent teeth with tooth position, gender and presence/absence of this disease; (3) evaluate the peptide profile in the saliva of patients with X-linked hypophosphatemic rickets using high performance liquid chromatography; and (4) characterize salivary proteins in this condition using unidimensional electrophoresis. On study 1, oral exams, laboratorial and histologic evaluations, cone-beam computed tomographies, panoramic and periapical radiographs were performed to properly institute the most adequate treatment strategy. On study 2, teeth were collected from 5 individuals from the same family. Gender, age, tooth position (anterior/posterior) and tooth type (deciduous/permanent) were recorded for each patient. Following collection, teeth were placed in 0.1% thymol solution until Micro-CT scan. Projection images were reconstructed and analyzed. On study 3, unstimulated whole and stimulated parotid saliva were obtained from 8 individuals with (AFF) and 8 healthy individuals, both genders, without (CON) x-linked hypophosphatemic rickets aged from 8 to 66 years. Supernatants were analyzed by high performance liquid chromatography, and the salivary flow rate (ml/min) was calculated. Each major peak in the HPLC chromatogram of each sample was characterized. On study 4, unstimulated whole and stimulated parotid saliva were also obtained, being total protein concentration determined by the Bicinchoninic Acid Protein (BCA) method. Proteins were characterized according to their molecular weights within the unidimensional electrophoresis. The study 1 showed the importance of the knowledge of clinical signs and symptoms of XLHR for the correct diagnosis of this disease, and for the establishment of preventive and comprehensive dental care. On article 2, teeth of all affected patients presented dentin with a different mineralization pattern compared to the teeth of the healthy individual with dentin defects observed next to the pulp chambers. On the third article, whole and parotid salivary flows were significantly different ($p = 0.001$), being flow of whole saliva higher (0.518 ± 0.282 mL/min) than parotid saliva (0.124 ± 0.086 mL/min). Whole salivary flow rate was higher in the AFF group (0.698 ± 0.229) than in the CON group (0.339 ± 0.210 mL/min) ($p = 0.006$). Twenty-eight peaks were found in whole and 21 peaks in parotid saliva. Whole saliva of the CON group presented lower number of peaks than AFF group. In parotid saliva, peaks 17 and 28 (retention times: 24 and 39 min) were found exclusively in the AFF group, and peak 13 (retention time: 19 min) exclusively in the CON. Article 4 showed difference concerning to total protein concentration between whole and parotid saliva ($p < 0.001$), being higher concentration found in whole saliva (102.603 ± 42.336 µg/mL) than in parotid saliva (0.699 ± 0.438 µg/mL). Bands with 102 kDa, 48 kDa and 24 kDa presented higher intensity in whole saliva of CON group ($p = 0.015$, $p = 0.043$ and $p = 0.022$). In conclusion, XLHR patients presented specific characteristics in dentin mineralization and salivary proteins and peptides, which can lead to differentiate these patients from healthy individuals, improving the diagnostic field.

Key-words: X-linked hypophosphatemic rickets; Micro-computed Tomography; Peptides; Saliva; Chromatography, High Pressure Liquid; Proteins; Electrophoresis

LISTA DE ABREVIATURAS

ADHR	Raquitismo hipofosfatêmico autossômico dominante
ASARM peptides	Peptídeos ricos em serina ácida e aspartato
AFF	Grupo afetado
BCA	Ácido bicinconínico
BSP	Sialoproteína óssea
CE	Grupo com experiência de cárie
CF	Grupo sem cáries
COMEPE	Comitê de ética em pesquisa
CON	Grupo controle
DMP1	Proteína 1 da matriz de dentina
DSPP	Sialofosfoproteína da dentina
ENS	Síndrome nevus epidermal
FGF-23	Fator de crescimento dos fibroblastos
FHR	Raquitismo hipofosfatêmico familiar
FS	Síndrome Fanconi
HHRH	Raquitismo hipofosfatêmico com fosfatúria
HIV	Vírus da imunodeficiência humana
HPLC	Cromatografia líquida de alta performance
MEPE	Fosfoglicoproteína da matriz extracelular
Micro-CT	Microtomografia computadorizada
NHERF1	Fator 1 regulador de troca sódio hidrogênio
NPT2a	Cotransportador 2a de fosfato de sódio
OGD	Displasia osteoglofônica
OPN	Oteopontina
PHEX	Gene regulador de fosfato com homologia para endopeptidases

PTH	Paratormônio
SDS-PAGE	Eletroforese em gel de poliacrilamida dodecil sulfato de sódio
SIBLING	Small integrin-binding ligand N-linked glycoprotein
TFA	Ácido trifluoroacético
TIO	Osteomalácia induzida por tumor
TMP/GFR	Limite máximo tubular de fosfato por taxa de filtração glomerular
Vit D	Vitamina D
XLHR	Raquitismo hipofosfatêmico ligado ao cromossomo X

SUMÁRIO

1 INTRODUÇÃO GERAL

O raquitismo é uma doença decorrente da falha de mineralização do osteóide e de ossos neoformados no esqueleto de crianças (SATTUR et al., 2010), que possui a hipofosfatemia como uma característica comum de todas as formas dessa doença, podendo ocorrer por deficiência de cálcio ou fósforo. Nas formas de raquitismo por deficiência de cálcio, a fosfatúria que leva a hipofosfatemia ocorre devido ao hiperparatireoidismo secundário. Já nas formas de raquitismo por deficiência de fósforo, essa característica é o defeito primário que resulta mais comumente da excreção renal aumentada de fosfato (JAGTAP et al., 2012) (Fig. 1).

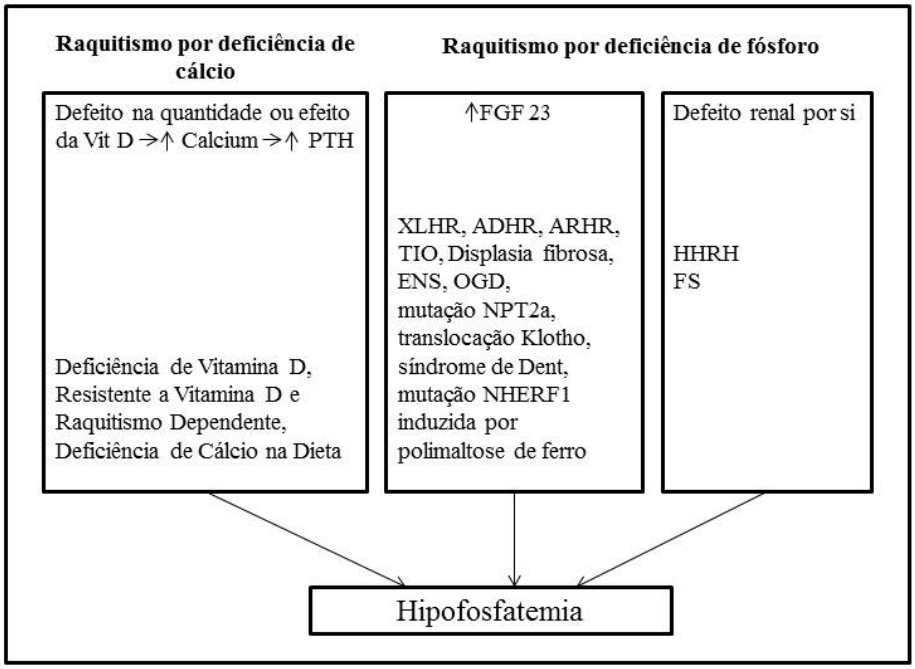


Figura 1: Classificação do raquitismo (ADHR: raquitismo hipofosfatêmico autossômico dominante, ENS: síndrome nevus epidermal, FGF 23: fator de crescimento dos fibroblastos, FS: síndrome Fanconi, HHRH: raquitismo hipofosfatêmico com fosfatúria, NPT2a: cotransportador 2a de fosfato de sódio, NHERF1: fator 1 regulador de troca sódio hidrogênio, OGD: displasia osteoglofônica, PTH: paratormônio, TIO: osteomalácia induzida por tumor, Vit D: vitamina D, XLHR: raquitismo hipofosfatêmico ligado ao cromossomo X).
Adaptado: Jagtap et al., 2012.

O raquitismo hipofosfatêmico familiar (OMIM#307800) é uma doença rara com incidência de 1 em 20.000 nascimentos (YONG; AIK, 2000), transmitida geralmente como uma característica dominante ligada ao cromossomo X. Mutações no gene regulador de fosfato com homologia para endopeptidases (PHEX) são responsáveis pela doença na maioria dos casos familiares (GAUCHER et al., 2009). As alterações bioquímicas e ósseas do raquitismo hipofosfatêmico ligado ao X (XLHR) são provavelmente causadas pela ação

aumentada do fator fosfatúrico decorrente da incapacidade do gene PHEX (Gene regulador de fosfato com homologia para endopeptidases) em inativar seu substrato, o que aconteceria em condições normais (Fig. 2) e por defeito primário dos osteoblastos (SOCIEDADE BRASILEIRA DE ENDOCRINOLOGIA E METABOLOGIA, 2004). Essa incapacidade do PHEX decorre geralmente de mutações inativantes, resultando em um gene não funcional. Consequentemente, os peptídeos ASARM (Peptídeos ricos em serina ácida e aspartato) acumulam-se impedindo a mineralização. Além disso, em condições normais, os peptídeos ASARM encontram-se ligados a proteína MEPE (Fosfoglicoproteína da matriz extracelular), mas por causa do PHEX defeituoso essas ligações são desfeitas e maior quantidade de peptídeos ASARM é liberada, potencializando, dessa maneira, a inibição da mineralização. O fator de crescimento dos fibroblastos, proteína FGF-23, também está presente nessa patogênese, inibindo a reabsorção tubular do fósforo (BOWE et al., 2001).

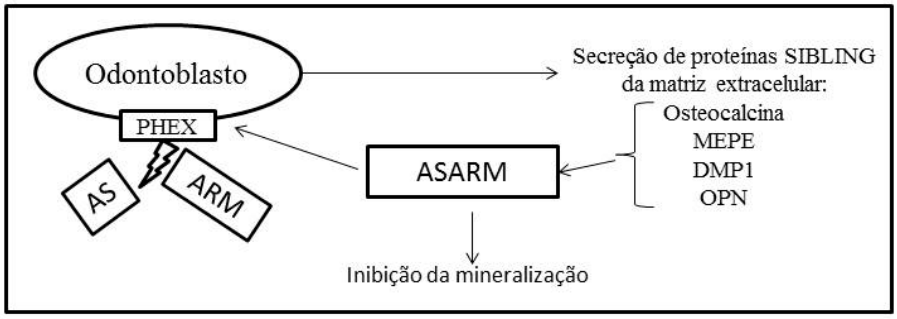


Figure 2: Ação dos peptídeos ASARM na mineralização. Em condições de normalidade, as proteínas SIBLING (Small integrin-binding ligand N-linked glycoprotein) que contém os peptídeos ASARM (inibidores da mineralização) são processadas por múltiplas enzimas proteolíticas liberando esses peptídeos na matriz extracelular, onde o PHEX tem ação neutralizadora, quebrando os peptídeos ASARM, permitindo, dessa maneira, a ocorrência normal da mineralização.
Adaptado: Jagtap et al., 2012.

Em exames laboratoriais observa-se hipofosfatemia, elevação da concentração plasmática de fosfatase alcalina, níveis normais de cálcio sérico, calciúria normal ou reduzida, redução de reabsorção tubular de fósforo, ausência de hiperparatireoidismo secundário e concentração plasmática de paratormônio (PTH) normal (GERTNER, 2003). Clinicamente, o paciente portador de XLHR caracteriza-se por apresentar baixa estatura, deformidades predominantemente em membros inferiores, alargamento metafisário, rosário raquítico e bossa frontal (ROOT; DIAMOND, 2002). Ao nascer, a criança apresenta-se com altura normal, sendo sua velocidade de crescimento reduzida ao longo dos primeiros anos de vida. O déficit de altura final é consequência do lento ritmo de crescimento que antecede o diagnóstico e a instituição terapêutica (ARICETA; LANGMAN, 2007; HAFFNER et al.,

2004; MÄKITIE et al., 2003). O diagnóstico diferencial entre o raquitismo hipofosfatêmico familiar e o “raquitismo por deficiência da vitamina D” é dado pela não observância de hipotonia, miopatia, tetania ou fraqueza muscular no XLHR (MUGHAL, 2002).

A principal característica clínica observada na cavidade oral é a formação de abscessos espontâneos recorrentes na ausência de cárie, ou trauma nas dentições decídua ou permanente (BATRA; TEJANI; MARS, 2006). Os dentes desses indivíduos caracterizam-se por apresentar mineralização dentinária com formação de amplas câmaras pulpares (SEOW, 2003), além da presença de grandes cornos pulpares e defeitos estruturais na dentina (MURAYAMA et al., 2000). McWhorter e Seale (1991) sugeriram que esses abscessos espontâneos são formados em decorrência da invasão bacteriana, em direção à polpa, através das microfendas existentes em esmalte e dentina. Cortes histológicos de dentes afetados mostram a desorganização dentinária (PEREIRA et al., 2004).

Radiograficamente, características dentárias específicas são comumente visualizadas por meio de radiografias periapicais (PEREIRA et al., 2004) ou panorâmicas (BARONCELLI et al., 2006; BATRA; TEJANI; MARS, 2006; MURAYAMA et al., 2000). Relatos prévios descreveram a avaliação de dentes decíduos e permanentes por meio de microscopia eletrônica de varredura e imunohistoquímica (CHAUSSAIN-MILLER et al., 2007; BOUKPESSI et al., 2006). Microtomografia computadorizada é um método radiográfico não invasivo de alta resolução com crescente utilização nos últimos anos em estudos sobre anatomia dentária (FARAH et al., 2010; ZHANG et al., 2009). Descrito em 1982 por Elliot e Dover, trata-se de um sistema microscópico baseado nos princípios de tomografia axial computadorizada, que promove distribuição de radiação para a secção de um objeto sólido sem a necessidade de realização de cortes. Essa técnica permite a análise microestrutural de pequenos órgãos, podendo-se visualizar vários parâmetros, como densidade mineral (FARAH et al., 2010), volume (IKRAM et al., 2009) e padrão de mineralização (ZHANG et al., 2009). Embora seja uma tecnologia de importância no estudo de microestruturas, não há relatos anteriores do uso da microtomografia computadorizada em dentes de pacientes diagnosticados com XLHR.

Algumas alterações proteicas foram previamente descritas nesses pacientes, tais como expressão de fosfoglicoproteína da matriz extracelular, marcadamente elevada (BRESLER et al., 2004), e presença anormal de complexos de proteínas de baixo peso molecular na estrutura dentária (BOUKPESSI et al., 2006). Esses componentes proteicos não têm sido pesquisados na saliva de pacientes diagnosticados com XLHR, embora o uso desse fluido como meio diagnóstico tenha avançado exponencialmente (STRECKFUS; BIGLER,

2002). O diagnóstico salivar é um campo dinâmico e emergente, utilizando nanotecnologia e diagnóstico molecular em doenças orais e sistêmicas (MALAMUD, 2011). A mais importante vantagem da coleta de saliva é a sua forma não invasiva e de fácil acesso. A possibilidade de identificação de um grande número de moléculas salivares, e a comparação dos níveis e expressão dessas substâncias com níveis sanguíneos tem tornado possível o estudo de marcadores microbiológicos (SASHIKUMAR; KANNAN, 2010), imunológicos (LEE et al., 2010), hormonais (TOUITOU et al., 2009), farmacológicos (HU et al., 2007) e oncológicos (SCARANO et al., 2010).

Peptídeos salivares têm sido relacionados com diferentes doenças (LAWRENCE, 2002). Giusti et al. (2007) encontraram diferenças no padrão de proteínas em saliva total humana de pacientes sadios e de pacientes diagnosticados com esclerose sistêmica difusa. Daep et al. (2006) caracterizaram a estrutura da inibição mediada por peptídeo do patógeno periodontal *Porphyromonas gingivalis* na formação do biofilme. Pacientes com doença celíaca também apresentaram diferenças no perfil de proteínas totais em saliva total humana (LENANDER-LUMIRAKI; IHALIN; LÄHTEENOJA, 2000). Alterações salivares foram pesquisadas em crianças com diabete insulino-dependente, mas não foram encontradas diferenças em relação a proteínas totais na saliva de pacientes afetados e dos controles (BELAZI et al., 1998). Nessas pesquisas, a coleta de saliva geralmente envolve saliva total (LENANDER-LUMIRAKI; IHALIN; LÄHTEENOJA, 2000) ou saliva da parótida (HOLBROOK; MOLAN, 1975), porém não há relatos prévios da diferença qualitativa e quantitativa em relação a conteúdo proteico salivar obtido através desses 2 métodos.

Diante do surgimento de tecnologias capazes de proporcionar avanços diagnósticos e terapêuticos no XLHR, tais como a microtomografia computadorizada e a análise salivar, e não havendo até a presente data estudos de peptídeos e proteínas salivares nesses indivíduos, torna-se necessária a realização de estudos com esse objetivo.

2 PROPOSIÇÃO

Os objetivos do presente trabalho foram:

2.1 Objetivo Geral

Estudar o perfil de peptídeos salivares e características orais e minerais dentárias em família de pacientes diagnosticados com raquitismo hipofosfatêmico familiar.

2.2 Objetivos Específicos

1. Descrever os principais achados orais e sistêmicos de uma família afetada por raquitismo hipofosfatêmico familiar e discutir tratamento eleito nesses casos.

2. Analisar o padrão de mineralização de esmalte e dentina em pacientes afetados por raquitismo hipofosfatêmico familiar, utilizando microtomografia computadorizada.

3. Apresentar o perfil de peptídeos presentes em saliva total e saliva de parótida em pacientes com e sem raquitismo hipofosfatêmico familiar.

4. Apresentar o perfil de proteínas presentes em saliva total e saliva de parótida em pacientes com e sem raquitismo hipofosfatêmico familiar.

3 CAPÍTULOS

Esta tese está baseada no Artigo 46 do Regimento Interno do Programa de Pós-graduação em Odontologia da Universidade Federal do Ceará que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato. Por se tratarem de pesquisas envolvendo seres humanos, ou partes deles, o projeto de pesquisa foi submetido à apreciação do Comitê de Ética em Pesquisa da Universidade Federal do Ceará, tendo sido aprovado sob o protocolo COMEPE nº 004/11, conforme o ofício nº 026/11 de 25 de fevereiro de 2011 (Anexo 1). Assim sendo, esta tese é composta de quatro capítulos, contendo artigos redigidos de acordo com as revistas científicas escolhidas (Anexos 2, 3 e 4), conforme descrito abaixo:

3.1. Capítulo 1

"Clinical Approach in Familial Hypophosphatemic Rickets: Report of Three Generations"

Autores: Eduardo Costa Studart Soares, Fábio Wildson Gurgel Costa, Thyciana Rodrigues Ribeiro, Ana Paula Negreiros Nunes Alves, Cristiane Sá Roriz Fonteles

Este artigo está publicado no periódico: ***Special Care in Dentistry*** (ISSN 1754-4505). **2012 Dec5. Doi: 10.1111/j.1754-4505.2012.00310.x (Epub ahead of print)**

3.2. Capítulo 2

"Different Patterns of Enamel and Dentin Mineralization Between Individuals with Familial Hypophosphatemic Rickets: a Micro-computed Tomography Study"

Autores: Thyciana R. Ribeiro, Fábio W. G. Costa, James C. Williams Jr., Cristiane S. R. Fonteles

Este artigo seguiu normas de publicação do periódico: ***Journal of Dental Research*** (ISSN 1544-0591)

3.3. Capítulo 3

"Salivary Peptide Profile of Patients with X-linked Hypophosphatemic Rickets Using High Performance Liquid Chromatography"

Autores: Thyciana R. Ribeiro, Karla S. S. Alves, Fábio W. G. Costa, Eduardo C. S. Soares, Manassés C. Fonteles, Cristiane S. R. Fonteles

Este artigo seguiu normas de publicação do periódico: ***The Journal of Clinical Endocrinology and Metabolism*** (ISSN 0021 972X)

3.4. Capítulo 4

"Comparison Between Whole and Parotid Salivary Protein Profile in a Family with X-linked Hypophosphatemic Rickets"

Autores: Thyciana R. Ribeiro, Cláudia F. Santos, Karla S. S. Alves, Fábio W. G. Costa, Eduardo C. S. Soares, Manassés C. Fonteles, Cristiane S. R. Fonteles

Este artigo seguiu normas de publicação do periódico: ***Journal of Dental Research*** (ISSN 1544-0591)

3.1 Capítulo 1

CLINICAL APPROACH IN FAMILIAL HYPOPHOSPHATEMIC RICKETS: REPORT OF THREE GENERATIONS

Abstract

Familial hypophosphatemic rickets is a hereditary disease characterized by the involvement of several family members, transmitted in most cases as an X-linked dominant trait. Oral manifestations can be the first evidences for an adequate and early diagnosis of X-linked hypophosphatemic rickets (XLHR). The present report describes the main systemic manifestations, oral findings and dental management in 3 generations of an affected family. Oral exams, laboratorial and histologic evaluations, cone-beam computed tomographies, panoramic and periapical radiographs were performed to properly institute the most adequate treatment strategy. The knowledge of clinical signs and symptoms of XLHR is essential for the correct diagnosis of this disease, and for the establishment of preventive and comprehensive dental care.

Keywords: dental treatment, oral medicine, rickets

INTRODUCTION

The early loss of deciduous teeth is an important issue among pediatric patients that must be appropriately addressed, since it may be a frequent sign of serious systemic derangements. Etiological factors of deciduous tooth loss include various syndromes, as well as hypophosphatemic disorders.[1] Familial hypophosphatemic rickets (FHR) is a condition generally transmitted as an X-linked dominant trait, which results from mutation of the PHEX gene , predominantly expressed in osteoblasts and odontoblasts.[2] X-linked hypophosphatemic rickets (XLHR) is rare with an approximate prevalence of 1 in 20,000 live births,[3] being characterized initially by delayed walking caused by deformity of the legs.[4] Oral manifestations, such as poorly mineralized dentin, enlarged pulp chambers and root canals, early tooth loss and/or painless spontaneous abscesses can be the first evidences for an adequate and early diagnosis of XLHR, thus dictating the importance of an appropriate clinical assessment by the dental professional.[4, 6-11] The aim of the present work was to describe the main systemic and oral findings of a family affected by XLHR, and to discuss the elected treatment strategy in these cases.

CASE REPORT

Six-year-old male patient presented with his mother and a 10-year-old sister to the Pediatric Dental Clinic (Federal University of Ceara, Brazil) with a chief complaint of recurrent gum abscesses without apparent cause, and premature tooth loss. Mother reported that herself, the child, and her 10-year old daughter were affected by XLHR (Fig. 1; Table 1). However, the child's older female sibling (13-year old) was not affected. Two XLHR-patients were in the mixed dentition stage, 2 had an incomplete set of permanent teeth, and 3 were edentulous. A previous history of tooth loss and formation of spontaneous gingival abscesses

(Fig. 2) was noted on all affected subjects. Only children presented abscesses and dental color alterations associated with the deciduous dentition.

The non-affected child presented unremarkable radiographic findings, whereas the 6-year-old boy showed a greater number of absent deciduous teeth and generalized bone loss (Fig. 3). Other characteristics consisted of advanced root resorption in the deciduous dentition, taurodontism and close proximity between pulp horns and dentin-enamel junctions (Fig. 3). Edentulism was a typical finding of adult patients (Figs. 5-7). In addition, dental caries, periradicular abscesses (Fig. 5) and poor demarcation of the lamina dura were observed in some teeth. Histologically, the lower left second deciduous molar of the proband showed an irregular and wide globular dentin, covered by enamel and invaded by microorganisms (Fig. 4).

Treatment was carefully planned according to age. In adults, many teeth were removed through conventional surgical technique. Patients with a partial or total edentulous ridge were referred for confection of dental prosthesis (Fig. 7). Pediatric patients were treated as part of a high-caries risk category. Treatment consisted of debridement therapy in the presence of periodontal disease, dental extractions in the absence of bone support or unfeasible endodontic treatment. Teeth with restorative needs were treated with composite materials and/or sealants. Space maintenance was instituted when needed. In case significant bone loss was present, teeth were only extracted when severe tooth mobility (> 3 mm) persisted. Thus, many caries-free teeth were lost during treatment due to severe periodontal disease. Short-term monitoring (every 3 months) was established to preserve the dentition and prevent further bone loss.

DISCUSSION

Low inorganic phosphate, high alkaline phosphatase, moderate or low calcium and normal levels of urea and creatinine, in addition to radiographic cuppind and fraying of metaphyses are typical characteristics of rickets.[12] However, the presence of these alterations alone may not be sufficient for proper differential diagnosis of this disease, requiring measurements of parathyroid hormone levels (PTH), 25-hydroxyvitamin D, tubular threshold maximum for phosphate per glomerular filtration rate (TMP/GFR), among other specific tests. Thus, differential diagnosis may include Blount disease, osteogenesis imperfecta, hypophosphatasia, skeletal dysplasia, chronic renal failure/renal osteodystrophy, deficient inorganic phosphate intake, vitamin-D deficiency, 25-hydroxylase deficiency, distal renal tubular acidosis, Fanconi syndromes, tumour-induced osteomalacia and dietary calcium deficiency.[12]

Hereditary hypophosphatemic rickets are primarily related to genetic defects, followed by metabolic alterations in vitamin D, phosphorus and phosphate, expressing specific oral findings that demand early diagnosis for adequate treatment and prevention.[5,7] Oral manifestations may vary as a function of family history, blood phosphate levels and patient's age at onset of therapy.[8] The development of spontaneous gingival abscesses, in the absence of tooth or periodontal-related diseases or injuries, has been indicated as the main diagnostic evidence of rickets, especially when the diagnosis of a systemic familial disease has not yet been confirmed.[9] Dental discoloration is often the first alteration noticed in children. In the present case report, this feature was frequently observed in association with recurrent gingival abscesses and premature deciduous tooth exfoliation, during early childhood. The present histologic alterations, including enamel micro-cracks, enlargement of the dentinal tubules, prominent and dysplastic interglobular dentin, enlarged pulp horns with close proximity to the

dentin-enamel junction[6,9] favors pulp necrosis in the deciduous and permanent dentitions, accelerating tooth loss.[10]

We believe that the dental professional should look for other diagnostic signs of rickets in periapical and panoramic radiographs. Hence, bone loss (especially in adults) and large pulp chambers (taurodontism) are findings commonly observed in first and second molars of male patients, who are severely affected by.[11] Presently, the number of affected individuals surpassed the number of unaffected patients. Moreover, male subjects were more severely affected than females. The only affected male child showed generalized taurodontism in molars (permanent and deciduous). Comparisons of radiographic findings between adults showed that men presented the highest rate of missing teeth and mineral bone loss.

The complexity of dental treatment in these patients usually demands careful evaluation.[8] Severe tooth loss led 5 adult patients to need prosthetic treatment, whereas 2 affected children required oral rehabilitation. In these instances, special attention was given to preventive procedures. Resin sealants were applied on all caries-free oclusal surfaces, for two different reasons: (1) attempting prevention of future spontaneous abscesses secondary to pulpal infection; (2) dental caries prevention. Many teeth were lost due to fast progression of periodontal disease, both in adults and children, especially in males. Ye et al.[13] suggested that patients affected by rickets were more susceptible to periodontal bone loss than a healthy population. Thus, some authors recommend early institution of preventive measures, including professional dental cleaning, topical application of fluoride varnish, pit and fissure sealants in the permanent dentition, resin composite coverage of the front teeth and stainless steel crown restorations in children. [8]

The present case report, showed the difficulties involved in the dental management of XLHR-patients. The need for long term oral health monitoring in pediatric and adult XLHR-patients is as important as the disease control performed by medical specialists. In these cases, a holistic approach should be the main focus by the dental professional, with evaluation and constant monitoring of related dental abnormalities to prevent intraoral abscesses, avoiding early loss of the primary and permanent dentitions, and its future consequences.

REFERENCES

1. Watanable K. Prepubertal periodontitis: a review of diagnostic criteria, pathogenesis, and differential diagnosis. J Periodontal Res 1990; 25:31-48.

2. Boukpessi T, Septier D, Bagga S, Garabedian M, Goldberg M, Chaussain-Miller C. Dentin alteration of deciduous teeth in human hypophosphatemic rickets. Calcif Tissue Int 2006; 79: 294-300.

3. Ichikawa S, Traxler EA, Estwick SA, Curry LR, Johnson ML, Sorenson AH, Imel EA, Econs MJ. Mutational survey of the PHEX gene in patients with X-linked hypophosphatemic rickets. Bone 2008; 43: 663-6.

4. Pereira CM, de Andrade CR, Vargas PA, Coletta RD, de Almeida OP, Lopes MA. Dental alterations associated with X-linked hypophosphatemic rickets. J Endod 2004; 30:241-5.

5. DiMeglio LA, White KE, Econs MJ. Disorders of phosphate metabolism. Endocrinol Metab Clin North Am 2000; 29:591-609.

6. Hillmann G, Geurtsen W, Hanover FRG. Pathohistology of undecalcified primary teeth in vitamin D-resistant rickets: review and report of two cases. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1996; 82:218-24.

7. Jan de Beur SM, Levine MA. Molecular pathogenesis of hypophosphatemic rickets. J Clin Endocrinol Metab 2002; 87:2467-73.

8. Douyere D, Joseph C, Gaucher C, Chaussain C, Courson F. Familial hypophosphatemic vitamin D-resistant rickets-prevention of spontaneous dental abscesses on primary teeth: a case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 107:525-30.

9. Murayama T, Iwatsubo R, Akiyama S, Amano A, Morisaki I. Familial hypophosphatemic vitamin D-resistant rickets: dental findings and histologic study of teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2000; 90:310-6.

10. Chaussain-Miller C, Sinding C, Septier D, Wolikow M, Goldberg M, Garabedian M. Dentin structure in familial hypophosphatemic rickets: benefits of vitamin D and phosphate treatment. Oral Dis 2007; 13:482-9.

11. Batra P, Tejani Z, Mars M. X-linked hypophosphatemia: dental and histologic findings. J Can Dent Assoc 2006; 72:69-72.

12. Rajah J, Thandrayen K, Pettifor JM. Clinical practice: diagnostic approach to the rachitic child. Eur J Pediatr 2011; 170: 1089-96.

13. Ye L, Liu R, White N, Alon US, Cobb CM. Periodontal status of patients with hypophosphatemic rickets – Case series. J Periodontol 2011, 82: 1530-5.

TABLE AND LEGENDS

Table 1. Biochemical exams (hematological and urinary) of the family members.

Table 1. Biochemical exams (hematological and urinary) of the family members.

	Subjects[a]							
	I-2	II-1	II-3	II-4	II-5	III-1	III-2	III-3
Age* (years)	64	33	31	29	27	13	10	6
Calcium[b] (mg/dL)	9.9	9.4	8.8	8.9	NI	8.1	9.6	9.4
Phosphorus[b] (mg/dL)	1.8	2.3	2.7	2.2	NI	5.3	2.9	2.4
Alkaline Phosphatase[b] (U/L)	130	237	181	265	NI	274	829	416
PTH[b] (pg/mL)	64.3	34.2	78.6	74.9	NI	19	34.7	43.3
Urinary Calcium[c] (mg/24h)	104	72	93	188	150	30	38	15
Serum Calcium[c] (mg/dL)	9.6	9.6	8.9	9.1	9.1	9.5	9.0	9.5
Urinary Phosphate[c] (mg/24h)	584	450	435	750	416	274	397	200
Serum Phosphate[c] (mg/dL)	2.2	2.4	2.5	2.3	2.2	4.8	3.0	2.3
Urinary Creatinine[c] (mg/24h)	813	828	849	1150	884	1049	408	382
Serum Creatinine[c] (mg/dL)	0.70	0.70	0.60	0.50	0.60	0.60	0.40	0.40
TMP/GFR[c] (mmol/L)	0.18	0.22	0.26	0.24	0.20	0.64	0.32	0.26

*Age at initial presentation
[a]Patients were identified according to the heredogram in Figure 1, where Roman numerals represent patient's generation and Arabic numerals identify the individual within a generation.
[b]Parameters measured at initial presentation.
[c]Parameters measured 3 years after presentation.
NI = not informed.
Reference values: Calcium (8.6–10.3), Phosphorus (children: 4–7 / adults: 2.5–5), Alkaline Phosphatase (age 4–6 years: <269 / age 7–12 years: <300 / adults: 34–104), PTH (10–69), Urinary Calcium (100–300), Serum Calcium (8.3–10.6), Urinary Phosphate (340–1000), Serum Phosphate (age 1–17 years: 3.0–7.0 / adults: 2.5–4.8), Urinary Creatinine (800–2000), Serum Creatinine (age 7–12 years: 0.5–1.0 / age >12 years, male: 0.70–1.30 / age >12 years, female: 0.60–1.10), TMP/GFR (0.80–1.35).

FIGURES LEGEND

Fig. 1. Pedigree of the reported family. The solid symbols indicate affected individuals. Roman numerals identify generations. Arabic numerals represent the individuals in each generation. In the second generation, there is one non-affected male stillbirth (II 6) and two deceased affected males who died at 2 months- age (II-7 and II-8) with unidentified cause of death.

Fig. 2. A) Presence of gingival abscess (white arrow) in the proband (III-3) related to right maxillary lateral incisor. B) PA radiography, indicating radiolucency (black arrow) associated with periodontal abscess.

Fig. 3. A) Panoramic radiography of the proband (age 6) showing large pulp chambers affecting both primary and permanent molars. B) Maxillomandibular cone-beam computed tomography of the upper first maxillary molar and lower first mandibular molar showing prominent pulp horns (black arrows) extending up to the dentin-enamel junction.

Fig. 4. A) Clinical lower left intra-oral view of the proband at initial presentation (age 6), demonstrating extensive gingival recession (white arrow) in the buccal region of the lower left primary second molar. B) Lower left primary second molar extracted secondary to periodontal disease. C) Photomicrography of the lower left primary second molar showing wide globular (black arrow) dentin containing tubules with microorganisms (HE, 100 μm, respectively).

Fig. 5. Panoramic radiography of the proband's mother (II-3) demonstrating multiple tooth loss, periapical radiolucency associated with the lower left permanent 3rd molar and bone loss associated with lower right permanent 3rd molar.

Fig. 6 A, B) Intra-oral and radiographic views of patient II-5 showing multiple tooth loss and bone loss associated with remaining molars.

Fig. 7 A, B) Prosthetic rehabilitation of patient II-4, edentulous since late adolescence.

Figure 1:

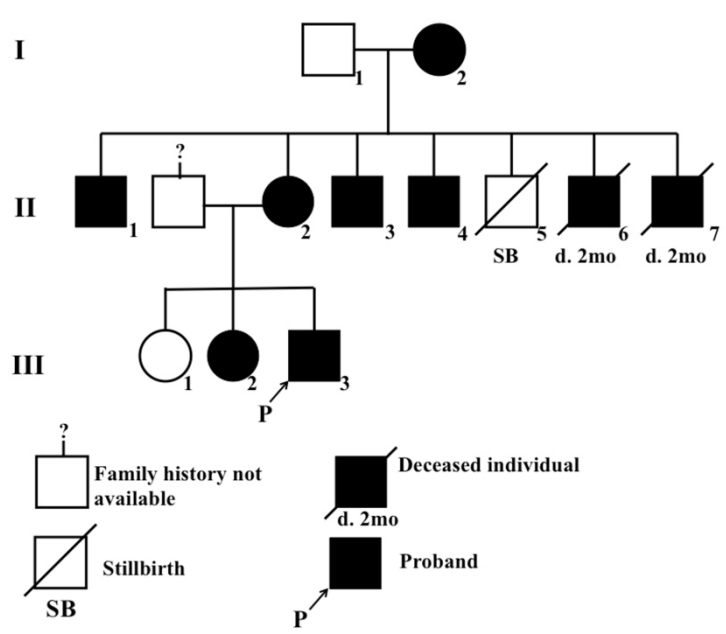

I
1
2
II
?
1
2
3
4
5
6
7
SB
d. 2mo
d. 2mo
III
1
2
3
P
?
Family history not available
Deceased individual
d. 2mo
Stillbirth
SB
Proband
P

Figure 2:

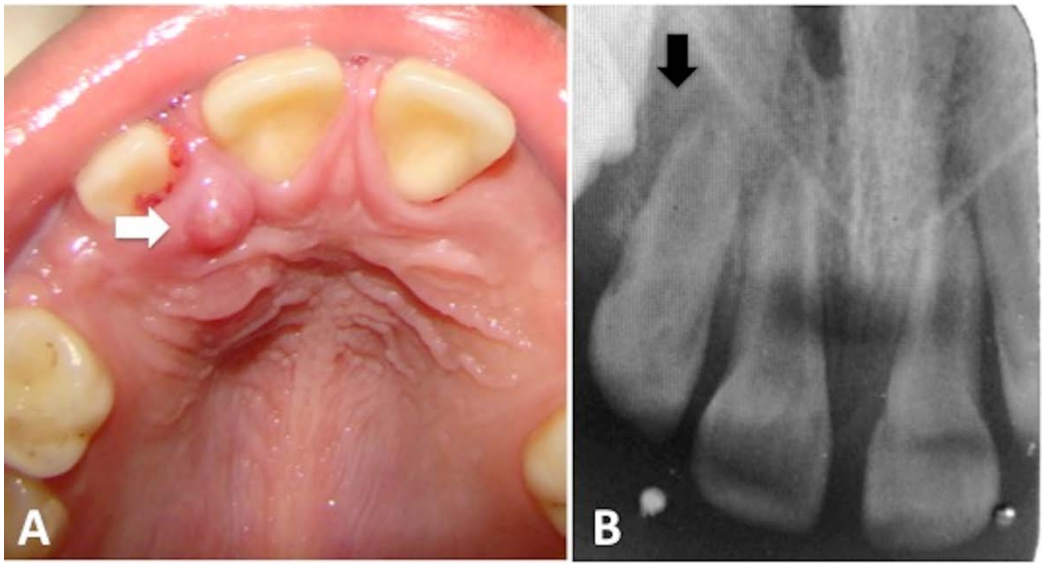


Figure 3:

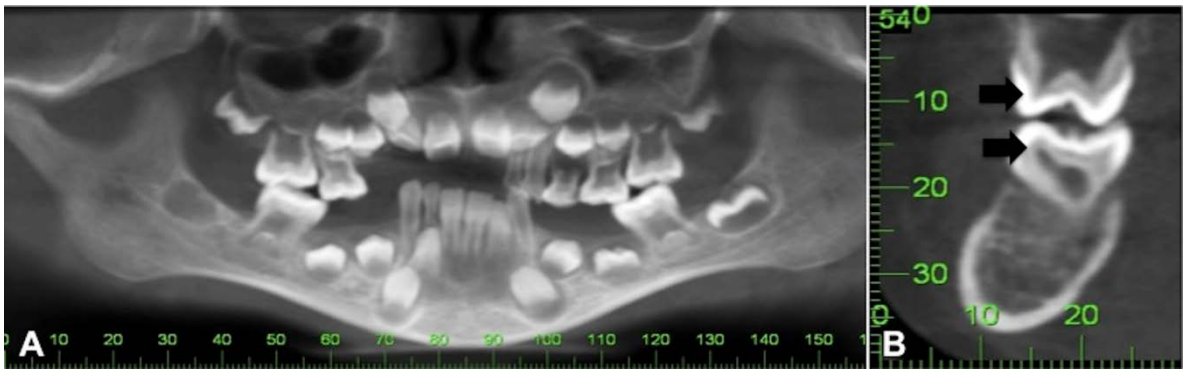


Figure 4:

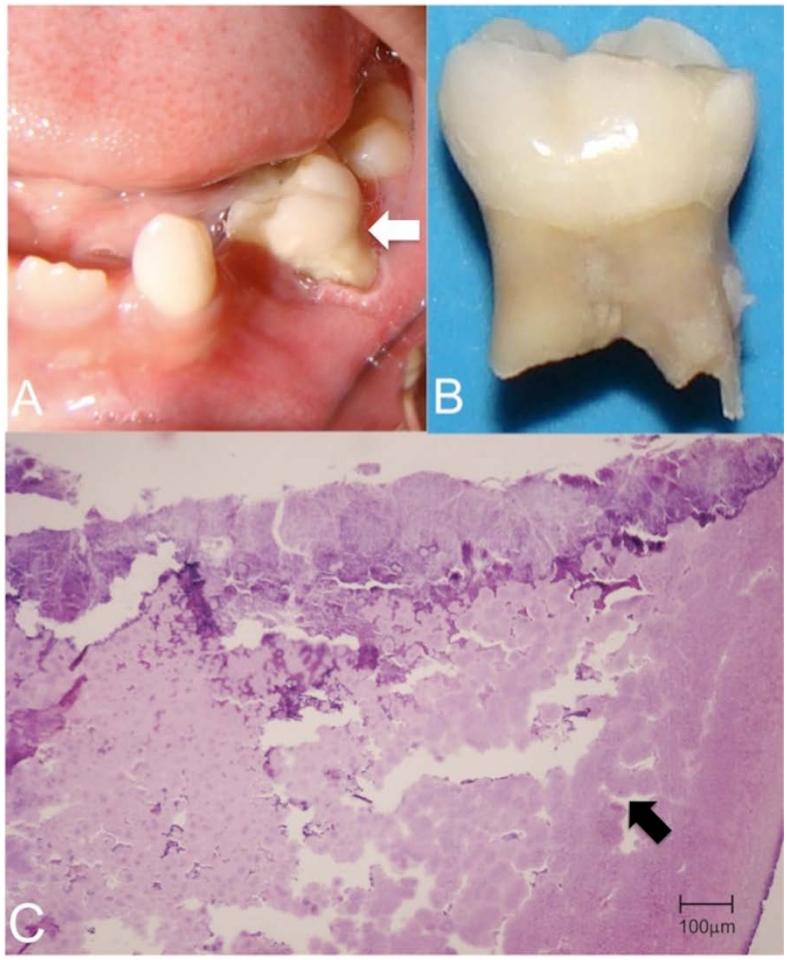


Figure 5:

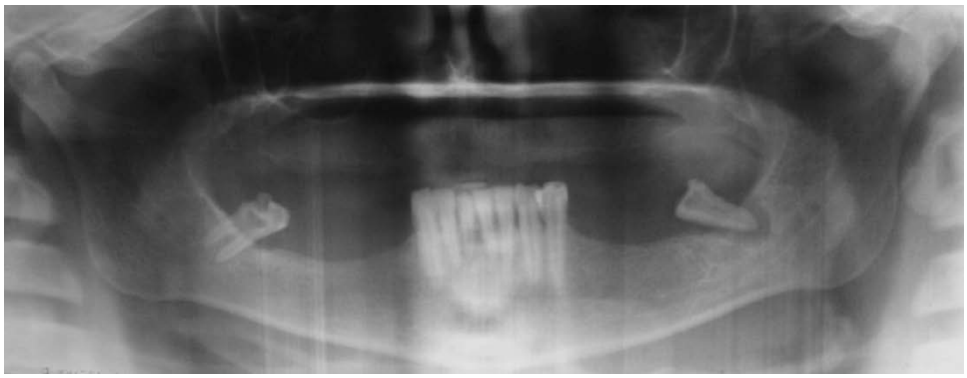


Figure 6:

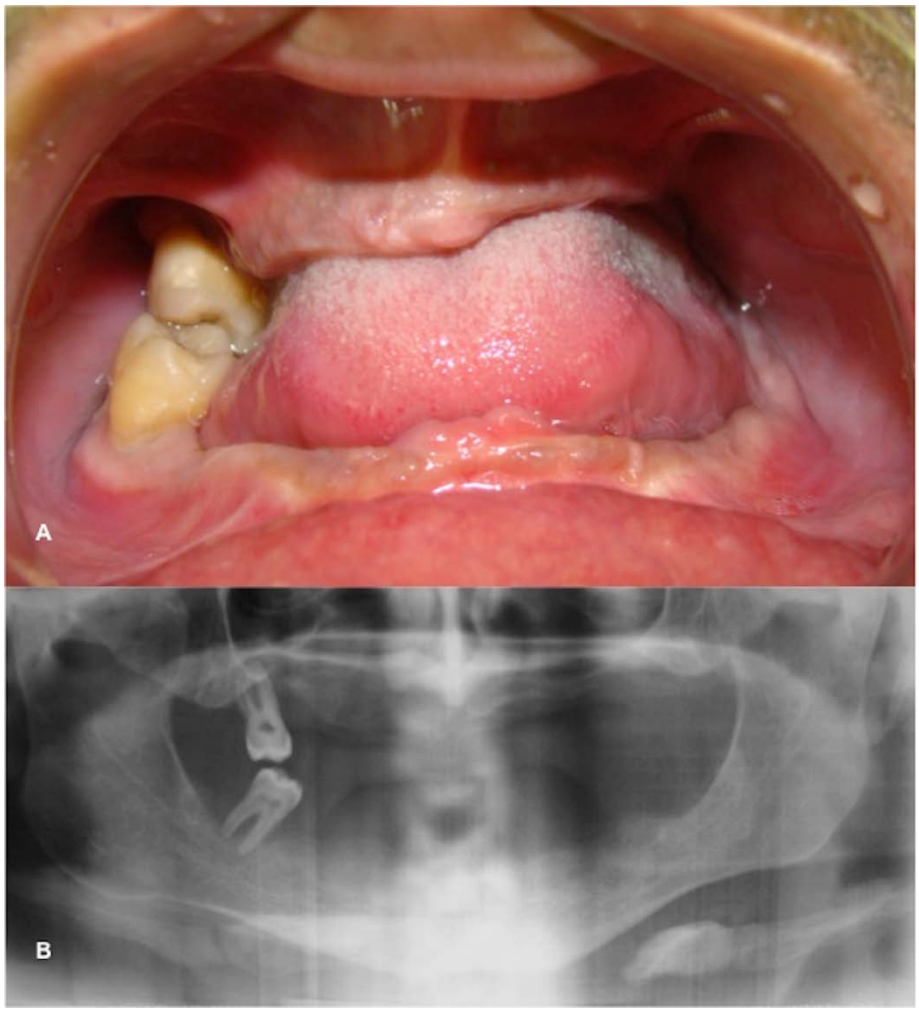
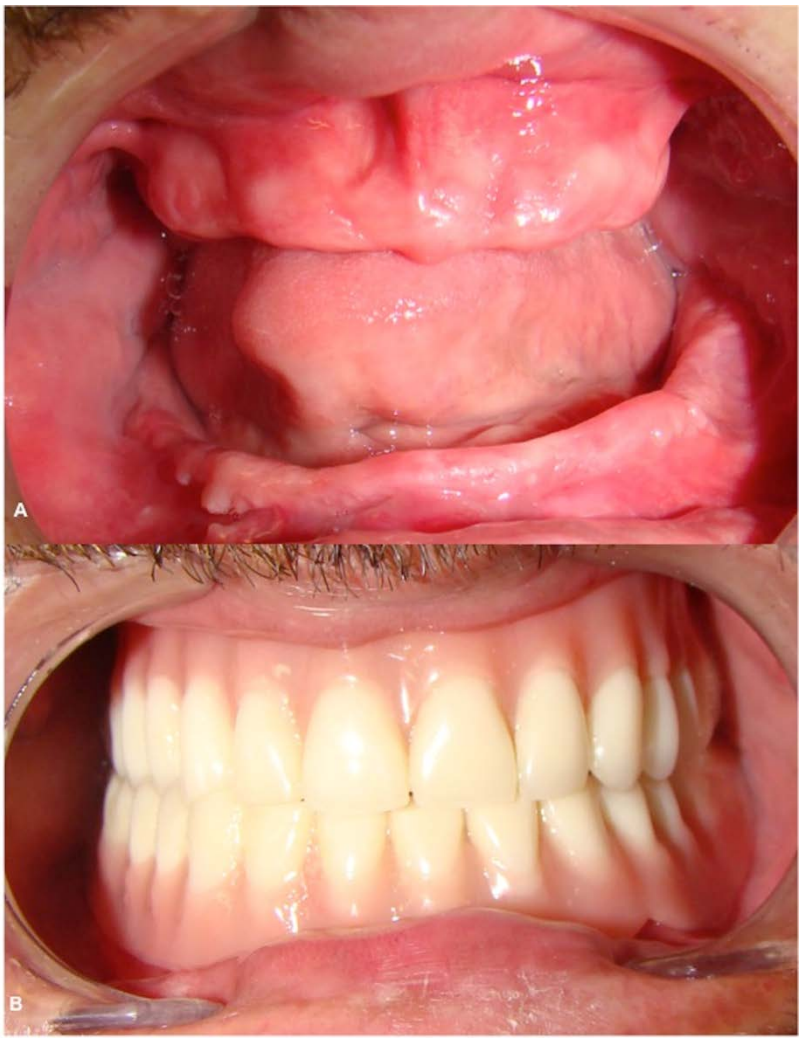


Figure 7:



3.2 Capítulo 2

DIFFERENT PATTERNS OF ENAMEL AND DENTIN MINERALIZATION BETWEEN INDIVIDUALS WITH FAMILIAL HYPOPHOSPHATEMIC RICKETS: A MICRO-COMPUTED TOMOGRAPHY STUDY

Abstract

The aim of the present study was to analyze the mineralization pattern of enamel and dentin in patients affected by X-linked hypophosphatemic rickets using micro-computed tomography (micro-CT), and to associate enamel and dentin mineralization in primary and permanent teeth with tooth position, gender and presence/absence of this disease.. Nineteen teeth collected from 5 individuals from the same family, being 1 non-affected and 4 affected by familial hypophosphatemic rickets. Gender, age, tooth position (anterior/posterior) and tooth type (deciduous/permanent) were recorded for each patient. Following collection, teeth were placed in 0.1% thymol solution until Micro-CT scan. Projection images were reconstructed and analyzed. A plot profile describing the gray-scale distance relationship in micro-CT images was achieved through a line bisecting each tooth in a region with presence of enamel and dentin. Furthermore the enamel and dentin mineralization density was measured and compared. Univariate Anova and Tukey tests were used for all comparisons. Teeth of all affected patients presented dentin with a different mineralization pattern compared to the teeth of the healthy individual with dentin defects observed next to the pulp chambers. Highly significant differences were found for gray values between anterior and posterior teeth ($p < 0.001$), affected and non-affected ($p < 0.001$), as well as when position and disease status were considered ($p < 0.001$). In conclusion, the mineralization patterns of dentin differed when comparing teeth from patients with and without familial hypophosphatemic rickets, mainly next to pulp chambers where areas with porosity and consequently lower mineral density and dentin defects were found.

Key words: X-linked hypophosphatemic rickets; Micro-computed Tomography; Teeth.

Introduction

Familial hypophosphatemic rickets (FHR) is a rare condition with an incidence of 1 in 20,000 births (Yong and Saw, 2000). Being generally transmitted as an X-linked dominant trait, mutations in the phosphate regulating gene with homologies to endopeptidases on the X-chromosome (PHEX) are responsible for the disease in most of the familial cases (Gaucher et al., 2009). Clinically, X-linked hypophosphatemic rickets (XLHR) is characterized by short stature, deformities predominantly in the lower limbs, metaphyseal widening, rachitic rosary and frontal bossing (Root and Diamond, 2002). At birth, children present normal height, being their growth rate reduced during the first year of life. The final deficit in height is a consequence of the slow speed of growth that precedes the diagnosis and therapeutic institution (Ariceta and Langman, 2007, Haffner et al., 2004; Mäkitie et al., 2003). The differential diagnosis from "vitamin D deficiency rickets" can be given by the non-compliance with hypotonia, myopathy, muscle weakness or tetany (Mughal, 2002).

The main oral clinical feature observed is the recurrence of spontaneous abscesses formation affecting multiple non-carious primary as well as permanent teeth (Batra et al., 2006, Soares et al., 2012). Teeth are characterized by deficient dentin mineralization with large pulp chambers (Seow, 2003), and the presence of large pulp horns and structural defects in the dentin (Murayama et al., 2000). McWhorter and Seale (1991) suggested that abscesses formation is due to bacterial invasion into the pulp through microcracks present in enamel and dentin. Histological sections of the affected teeth show dentin disorganization (Pereira et al., 2004). Radiographically, dental characteristics are generally examined through periapical (Pereira et al., 2004) or panoramic radiographs (Baroncelli et al., 2006; Batra et al., 2006; Murayama et al., 2000). Primary and permanent teeth were also evaluated using scanning electron microscopy and immunohistochemistry (Chaussain-Miller et al., 2007; Boukpessi et al., 2006).

X-ray micro-computed tomography (micro-CT) is a high resolution, non-destructive radiographic method, which has been increasingly used in studies of dental anatomy (Farah et al., 2010; Zhang et al., 2009). Described by Elliot and Dover (1982), micro-CT is a microscopic system based on the principles of computed tomography, which promotes radiation distribution to the section of a solid object, without the need to make cuts. This technique allows microstructural analysis of small bodies, enabling the study of various parameters such as mineral density (Farah et al., 2010), volume (Ikram et al., 2009) and mineralization pattern (Zhang et al., 2009, McKee et al., 2013). Although micro-CT is an important technology in microstructure studies, no previous work has reported its use in teeth

of patients diagnosed with FHR. The aim of the present study was to analyze the mineralization pattern of enamel and dentin in a family of patients affected by FHR using micro-CT, and to verify variations in enamel and dentin mineralization in primary and permanent teeth, as a function of tooth position, gender and presence/absence of this disease.

Materials and Methods

Samples

Nineteen teeth collected from 5 individuals from the same family, being 1 non-affected and 4 affected by FHR, were used for this study. These teeth were previously extracted because of the presence of periodontal disease or dental caries and they were being kept by the patients prior to their participation in the study. The following characteristics were recorded for each patient: gender, age, tooth position (anterior/posterior) and tooth type (deciduous/permanent) (Table 1). Following collection, teeth were placed in 0.1% thymol solution until Micro-CT scan. This study was approved by the Ethics Committee of the Federal University of Ceará Medical School (Brazil) (protocol # 004/11). Consent and tooth donation forms were signed by volunteers or legal guardians, prior to patient enrollment in the study.

Micro-CT analysis

High-resolution micro-CT (SkyScan 1172; SkyScan, Kontich, Belgium) was used to scan all teeth. Samples were wrapped in Parafilm (Brand, Germany) as a way to prevent drying, and affixed to the scanning stage by use of this same plastic paraffin film. Projection images were obtained by the use of the following scanner settings: voltage 70kV, resolution 8.9μm, 0.5mm aluminium filter, stage rotation 0.4^{o}/s and frame averaging 2. Flat-field corrections were used to minimize background noise. Projection images were reconstructed by use of manufacturer-provided software (NRecon; SkyScan) with the following settings: beam hardening 33, ring reduction 5 and threshold 0.00 to 0.13. In addition, post-alignment was optimized separately for each specimen.

Reconstructed images were analyzed with the ImageJ 1.43u software (Wayne Rasband, National Institutes of Health, USA). A plot profile describing the gray-scale distance relationship in micro-CT images was achieved through a line bisecting each tooth at a standardized cross-sectional oclusal level with presence of enamel and dentin. In each tooth, 5 lines were drawn and the average of gray-scale values for enamel and dentin was calculated. Enamel and dentin mineralization density was measured and compared.

Statistics

All statistical tests were performed with SPSS 9.0 software. Univariate Anova was used for all comparisons, with either enamel or dentin gray-scale as the dependent variable, and variables such as gender, age, tooth position, tooth type and presence/absence of FHR as the independent variables. Post hoc tests were only used when more than 2 groups were being compared. In these cases, Tukey test was applied.

Results

Teeth of all affected patients presented dentin with a different mineralization pattern compared to the teeth of the healthy individual (Fig. 1). Dentin defects were observed next to the pulp chambers as areas with porosity and consequently lower mineral density. No visual differences were detected in enamel and pulp spaces. Distinctive mineralization patterns of each tooth are shown in figure 2.

When considering tooth position (anterior – incisor, canine vs. posterior – premolar, molar) and presence/absence of FHR, comparisons of enamel gray-scale values differed only between anterior and posterior teeth ($p = 0.027$), but no difference was found between affected and non-affected individuals ($p = 0.965$) (Fig. 3), nor when all these variables were considered ($p = 0.823$). However, when these comparisons were done with dentin gray-scale value as the dependent variable, highly significant differences were found for gray values between anterior and posterior teeth ($p < 0.001$), affected and non-affected ($p<0.001$) (Fig. 4), as well as when position and disease status were considered ($p < 0.001$).

By evaluating dentin gray-scale values and gender alone, a significant difference between males and females ($p < 0.001$) was verified; having the female gender highest values of gray-scale, but this difference was not noted when comparing enamel gray-scale values between genders ($p = 0.057$). Tooth loss was highest among males. Dentin gray values differed between dentitions ($p = 0.008$); hence, deciduous teeth presented higher gray-scale values than permanent teeth.

Discussion

Micro-CT is a miniaturized version of medical CT and has been used extensively for in vitro dental research (Kühnisch et al., 2012; Nakata et al., 2012; Cochrane et al., 2012; Qi et al., 2012). The technique allows three-dimensional analyses of both structure and density (or concentration), the latter requiring prior knowledge of composition (Davis et al., 2013). However micro-CT was not been previously used to assess the mineral structure of

teeth from rickets patients. In addition, scarce information about micro-CT analysis in experimentally induced X-linked hypophosphatemia is available (Salmon et al., 2013). Other studies have focused on the tooth mineral structure from these patients using scanning electron microscopy (Boukpessi et al., 2006; Seow and Perham, 1990), transmission electron microscopy (McKee et al., 2011), immunohistochemical examination (Chaussain-Miller et al., 2007; Boukpessi et al., 2006) and radiographic examination (Pereira et al., 2004).

Teeth of all affected patients presented dentin with a different mineralization pattern compared to teeth of the healthy individual, being dentin defects observed next to pulp chambers as areas with porosity and consequently lower mineral density. This fact was confirmed by dentin gray-scale values, which differed between affected and non-affected patients Although odontoblastic function is normal in XLHR patients, dental mineralization is inadequate. Hence, hypophosphatemia generates a dysplastic and poorly mineralized dentin with areas of interglobular dentin (Murayama et al., 2000). Interglobular dentin and irregular dentinal tubules were histologically observed by Pereira et al (2004). These dentin defects and pulp chambers abnormalities were also observed in a tooth model of X-linked hypophosphatemia through micro-CT assessment (Salmon et al., 2013). In the present study, no visual differences were detected in enamel mineralization and pulp chamber. Enamel gray-scale did not differ between affected and non-affected individuals. Harris and Sullivan (1960), Archard and Witkop (1966) also described the enamel as normal but thin. However enamel hypoplasia has been reported in several studies (Murayama et al., 2000; Herbert 1986; Ozkan et al., 1984). Large canals and radicular chambers were also previously described (Pereira et al., 2004). These abnormalities may explain the common outbreak of periradicular abscesses in the absence of caries or history of trauma, typically found in XLHR, caused by entry of bacteria via enamel microfractures extending to pulp leading to tissue necrosis.

Enamel and dentin gray-scales differed between anterior (incisive and canine) and posterior (premolar and molar) teeth in the presence and absence of the disease. This feature was observed by Sánchez-Quevedo et al. (2001) when human teeth with amelogenesis imperfecta were evaluated through scanning electron microscopy and X-ray microprobe analysis. The authors analyzed dental fragments from members of a family clinically and genetically diagnosed as having amelogenesis imperfecta to establish the morphological patterns and the quantitative concentration of calcium in the enamel of anterior and posterior teeth. Calcium levels in the enamel of teeth with and without amelogenesis imperfecta differed significantly between anterior and posterior teeth, indicating that the factors that

influence normal mineralization in different regions of the dental arch are not altered in the process of amelogenesis imperfecta (Sánchez-Quevedo et al., 2001).

In this study, a higher mineral density was observed in female patients affected by rickets when compared with males. We believe that this observation should be justified by a disease phenotype with different penetrance among studied individuals and especially between the sexes. Shields et al. (1990) observed that the area of the pulp chamber of teeth in individuals affected by XLHR was higher than in the control group who did not have the disease, and the mean value was statistically higher in men than in women. If we consider that the area of the pulp chamber is inversely proportional to the amount of surrounding dentin, it can be deduced with their study that women affected by the disease had a higher content of dentin than men and, thus, these findings may corroborate those found in the present microtomographic study.

Deciduous teeth expressed the highest gray-scale values, suggesting a higher mineralization pattern in the dentin of deciduous teeth compared to permanent teeth. This finding is consistent with the study of Hirayama et al. (1986) that reported peritubular dentin thickness, which is the dentin with higher mineral content, 2 to 5 times greater in deciduous teeth, with values ranging from 3 to 20 micrometers when compared to permanent teeth. Also supporting our finding, Sumikawa et al. (1999) reported that the numerical density of dentinal tubules was higher in deciduous than in permanent teeth, being observed decrease in the area of intertubular dentin and, thereby increase of peritubular dentin.

Enamel gray-scale values did not differ between deciduous and permanent dentition which differs from a study that compared mineralization differences between human deciduous and permanent enamel measured by quantitative microradiography. In this study, the authors found lower overall mineralization in the deciduous dentition and lower mineral levels in deciduous enamel (Wilson, Beynon, 1989).

In conclusion, the mineralization patterns of dentin in teeth from patients affected by FHR differed from teeth of healthy patients in an affected family. This finding is better noted next to pulp chambers where areas with porosity and consequently lower mineral density and dentin defects were observed. The utilization of micro-CT was of great importance to the visualization of these micro defects, that were described previously, but not with the resolution and details showed in the present work.

References

Archard HO, Witkop CJ Jr (1966). Hereditary hypophosphatemia (vitamin D-resistant rickets) presenting primary dental manifestations. *Oral Surg Oral Med Oral Pathol* 22: 184-193.

Ariceta G, Langman CB (2007). Growth in x-linked hypophosphatemic rickets. *Eur J Pediatr* 166: 303-309.

Baroncelli GI, Angiolini M, Ninni E, Galli V, Saggese R, Giuca MR (2006). Prevalence and pathogenesis of dental and periodontal lesions in children with x-linked hypophosphatemic rickets. *Eur J Paediatr Dent* 7: 61-66.

Batra P, Tejani Z, Mars M (2006). X-Linked hypophosphatemia dental and histologic findings. *JCDA* 72: 69-72.

Boukpessi T, Septier D, Bagga S, Garabedian M, Goldberg M, Chaussain-Miller C (2006). Dentin alterations of deciduous teeth in human hypophosphatemic rickets. *Calcif Tissue Int* 79: 294-300.

Chaussain-Miller C, Sinding C, Septier D, Wolikow M, Goldberg M, Garabedian M (2007). Dentin structure in familial hypophosphatemic rickets: benefits of vitamin D and phosphate treatment. *Oral Dis* 13: 482-489.

Cochrane NJ, Anderson P, Davis GR, Adams GG, Stacey MA, Reynolds EC (2012). An X-ray microtomographic study of natural white-spot enamel lesions. *J Dent Res* 91: 185-191.

Davis GR, Everhed AN, Mills D (2013). Quantitative high contrast X-ray microtomography for dental research. J Dent 41: 475-482.

Elliott JC, Dover SD (1982). X-ray microtomography. *J Microsc* 126: 211-213.

Farah RA, Swain MV, Drummond BK, Cook R, Atieh M (2010). Mineral density of hypomineralised enamel. *J Dent* 38: 50-58.

Gaucher C, Walrant-Debray O, Nguyen TM, Esterle L, Garabédian M, Jehan F (2009). PHEX analysis in 118 pedigrees reveals new genetic clues in hypophosphatemic rickets. *Hum Genet* 125: 401-411.

Haffner D, Nissel R, Wühl E, Mehls O (2004). Effects of growth hormone tretament on body proportions and final height among small children with x-linked hypophosphatemic rickets. *Pediatrics* 113: e593-e596.

Harris R, Sullivan HR (1960). Dental sequelae in deciduous dentition in vitamin D resistant rickets: Case report. *Aust Dent J* 5: 200-203.

Herbert FL (1986). Hereditary hypophosphatemia rickets: an important awareness for dentists. *ASDC J Dent Child* 53: 223-226.

Hirayama A, Yamada M, Miake K (1986). An electron microscopy study on dentinal tubules of human deciduous teeth. *Shikwa Gakuho* 86: 1021-1031.

Ikram OH, Patel S, Sauro S, Mannocci F (2009). Micro-computed tomography of tooth tissue volume changes following endodontic procedures and post space preparation. *Int Endod J* 42: 1071-1076.

Kühnisch J, Galler M, Seitz M, Stich H, Lussi A, Hickel R, Kunzelmann KH, Bücher K (2012). Irregularities below the enamel-dentin junction may predispose for fissure caries. *J Dent Res* 91: 1066-1070.

Mäkitie O, Doria A, Kooh SW, Cole WG, Daneman A, Sochett E (2003). Early tretament improves growth and biochemical and radiographic outcome in X-linked hypophosphatemic rickets. *J Clin Endocrinol Metab* 88: 3591-3597.

McKee MD, Nakano Y, Masica DL, Gray JJ, Lemire I, Heft R, Whyte MP, Crine P, Millán JL (2011). Enzyme replacement therapy prevents dental defects in a model of hypophosphatasia. *J Dent Res* 90: 470-474.

McKee MD, Yadav MC, Foster BL, Somerman MJ, Farquharson C, Millán JL (2013). Compunded PHOSPHO1/ALPL deficiencies reduce dentin mineralization. *J Dent Res* (Epub ahead of print).

McWhorter AG, Seale NS (1991). Prevalence of dental abscesses in a population of children with vitamin D resistant rickets. *Pediatr Dent* 13: 91-96.

Mughal Z (2002). Rickets in childhood. *Semin Musculoskelet Radiol* 6: 183-190.

Murayama T, Iwatsubo R, Akiyama S, Amano A, Morisaki I (2000). Familial hypophosphatemic vitamin D resistant rickets: dental findings and histology study of teeth. *Oral Surg Oral Med Oral Patol Oral Radiol Endod* 90: 310-316.

Nakata K, Nikaido T, Nakashima S, Nango N, Tagami J (2012). An approach to normalizing micro-CT depth profiles of mineral density for monitoring enamel remineralization progress. *Dent Mater* 31: 533-540.

Ozkan S, Ucok Z, Alagol F (1984). Dental manifestations of familial hypophosphatemic vitamin-D-resistant rickets. *ASDC J Dent Child* 51: 448-450.

Pereira CM, Andrade CR, Vargas PA, Coletta RD, Almeida OP, Lopes MA (2004). Dental alterations associated with x-linked hypophosphatemic rickets. *J Endod* 30: 241-245.

Qi YP, Li N, Primus CM, Ling JQ, Pashley DH, Tay FR (2012). Remineralization of artificial dentinal caries lesions by biomimetically modified mineral trioxide aggregate. *Acta Biomater* 8: 836-842.

Root AW, Diamond FB Jr (2002). Disorders of calcium metabolism in the child and adolescent. In: Sperling MA, ed. Pediatric endocrinology. 2nd ed. Philadelphia: Saunders: 650-651.

Salmon B, Bardet C, Khaddam M, Naji J, Coyac BR, Baroukh B, Letourneur F, Lesieur J, Decup F, Le Denmat D, Nicoletti A, Pollard A, Rowe PS, Huet E, Vital SO, Linglart A, McKee MD, Chaussain C (2013) MEPE-derived ASARM peptide inhibits odontogenic differentiation of dental pulp stem cells and impairs mineralization in tooth models of X-linked hypophosphatemia. *Plos one* 8: e56749.

Sánchez-Quevedo MC, Ceballos G, Garcia JM, Rodrígues IA, Gómez de Ferraris ME, Campos A (2001). Scanning electron microscopy and calcification in amelogenesis imperfect in anterior and posterior human teeth. *Histol Histopathol* 16: 827-832.

Seow WK, Perham S (1990). Enamel hypoplasia in prematurely-born children: a scanning electron microscopic study. *J Pedod* 14: 235-239.

Seow WK (2003). Diagnosis and management of unusual dental abscesses in children. *Aust Dent J* 48: 156-168.

Shields ED, Scriver CR, Reade T, Fujiwara TM, Morgan K, Ciampi A, Schwartz S (1990). X-linked hypophosphatemia: The mutant gene is expressed in teeth as well as in kidney. *Am J Hum Genet* 46: 434-442.

Soares EC, Costa FW, Ribeiro TR, Alves AP, Fonteles CS (2012). Clinical approach in familial hypophosphatemic rickets: report of three generations. *Spec Care Dentist* (Epub ahead of print).

Sumikawa DA, Marshall GW, Gee L, Marshall SJ (1999). Microstructure of primary tooth dentin. *Pediatr Dent* 21: 439-444.

Wilson PR, Beynon AD (1989). Mineralization differences between human deciduous and permanent enamel measured by quantitative microradiography. *Arch Oral Biol* 34: 85-88.

Yong SM, Saw A (2000). X-Linked hypophosphatemic rickets – A report of 2 cases and review of literature. *Med J Malysia* 55: 101-104.

Zhang X, Rahemtulla F, Zhang P, Li X, Beck P, Thomas HF (2009). Normalization of calcium status reverses the phenotype in dentin, but not in enamel of VDR-deficient mice. *Arch Oral Biol* 54: 1105-1110.

Tables:

Table 1: Teeth distributed by gender, age and presence/absence of X-linked hypophosphatemic rickets of study volunteers

Individuals	FHR	Gender	Age (years)	Tooth	Tooth position	Tooth type
1	Absent	Female	13	1	Posterior	Deciduous
				2	Anterior	Deciduous
				3	Posterior	Deciduous
				4	Anterior	Deciduous
				5	Posterior	Deciduous
				6	Posterior	Deciduous
2	Present	Male	6	1	Anterior	Deciduous
				2	Posterior	Deciduous
				3	Anterior	Deciduous
				4	Anterior	Deciduous
				5	Anterior	Deciduous
				6	Posterior	Deciduous
				7	Anterior	Deciduous
				8	Anterior	Deciduous
				9	Anterior	Deciduous
3	Present	Female	10	1	Posterior	Deciduous
4	Present	Female	31	1	Posterior	Permanent
5	Present	Male	27	1	Posterior	Permanent
				2	Anterior	Permanent

Figures:

Figure 1: Two-dimensional micro-CT reconstructed images (Progam: Nrecon-Skyscan). Cross-section at a more oclusal level showing normal pattern of mineralization in non-affected individuals (A, molar; B, incisor). Cross-section at a more oclusal level showing wider spread hypomineralization defects in dentin of affected individual (C, molar; D, dentin).

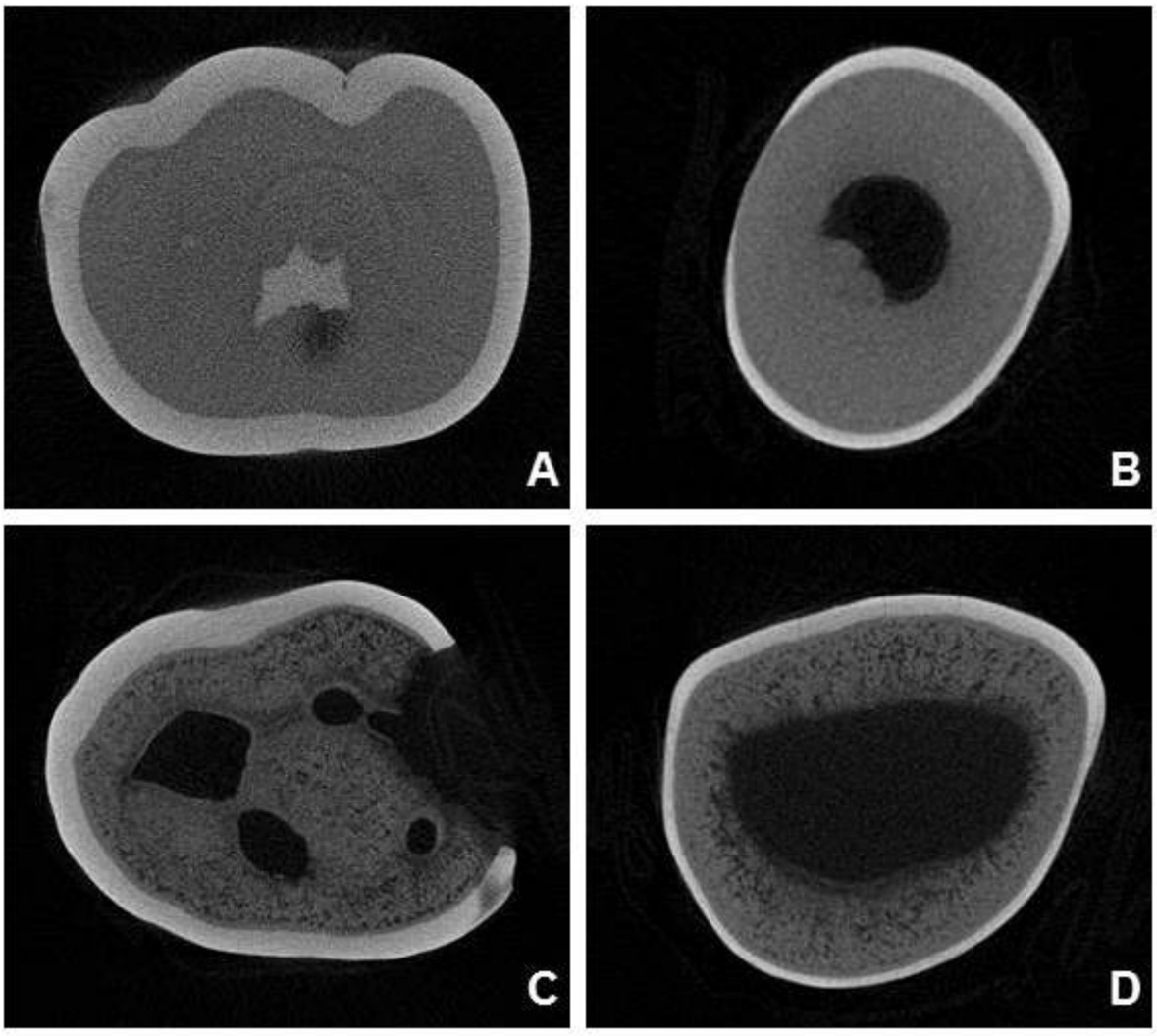


Figure 2: Enamel and dentin mineral density distribution histograms in affected and non-affected individuals. Direction of histograms: (1) from left to right = from enamel to pulp (individual 1, teeth 1, 2, 3, 4 and 5; individual 2, teeth 1, 2, 3, 4, 5, 6, 7 and 9; individual 4, tooth 1; individual 5, tooth 1 and individual 5, tooth 2). (2) from left to right = from pulp to enamel (individual 1, tooth 6; individual 2, tooth 8 and individual 3, tooth 1)

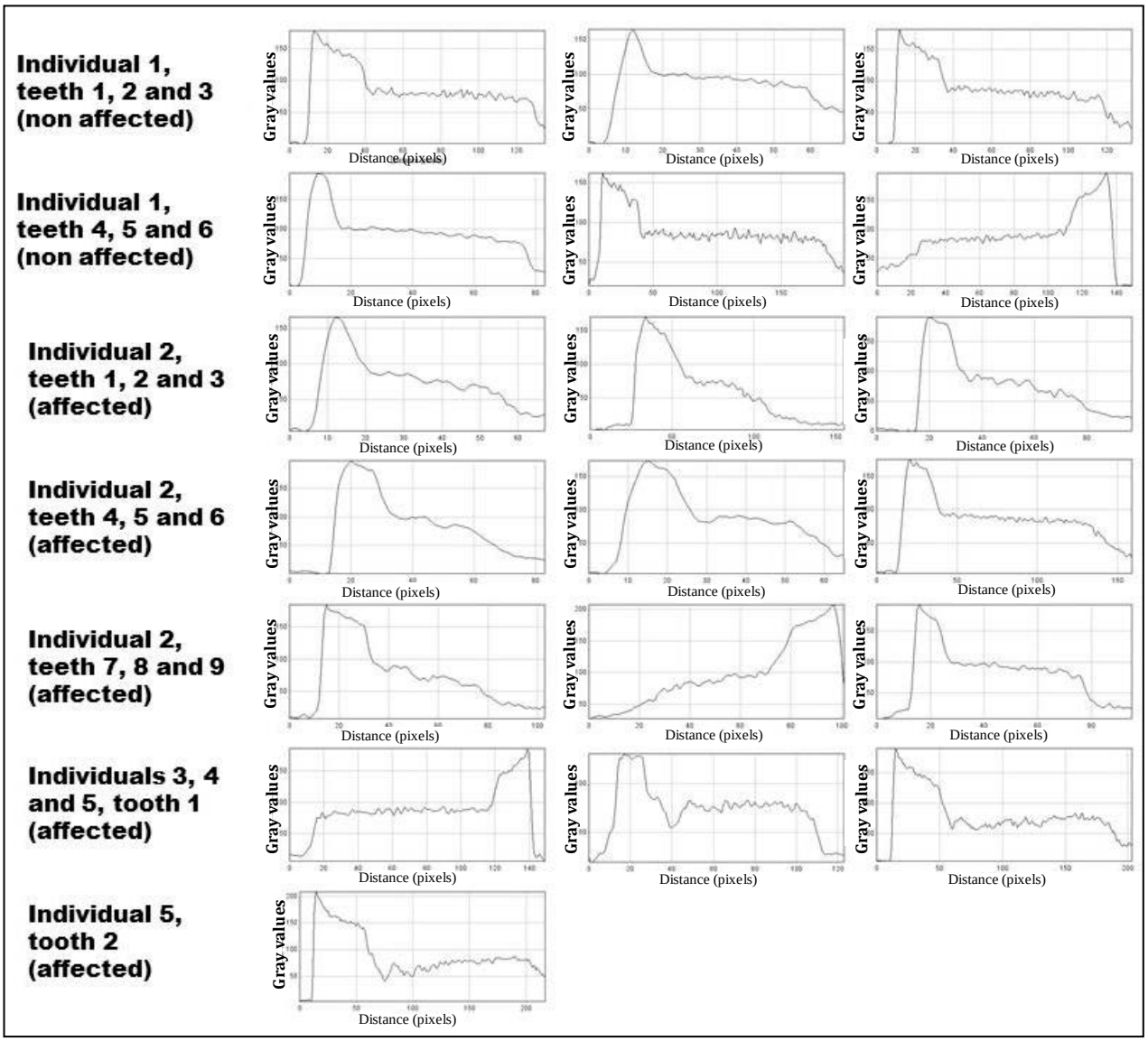


Figure 3: Comparison of enamel gray-scale values between affected and non-affected individuals .

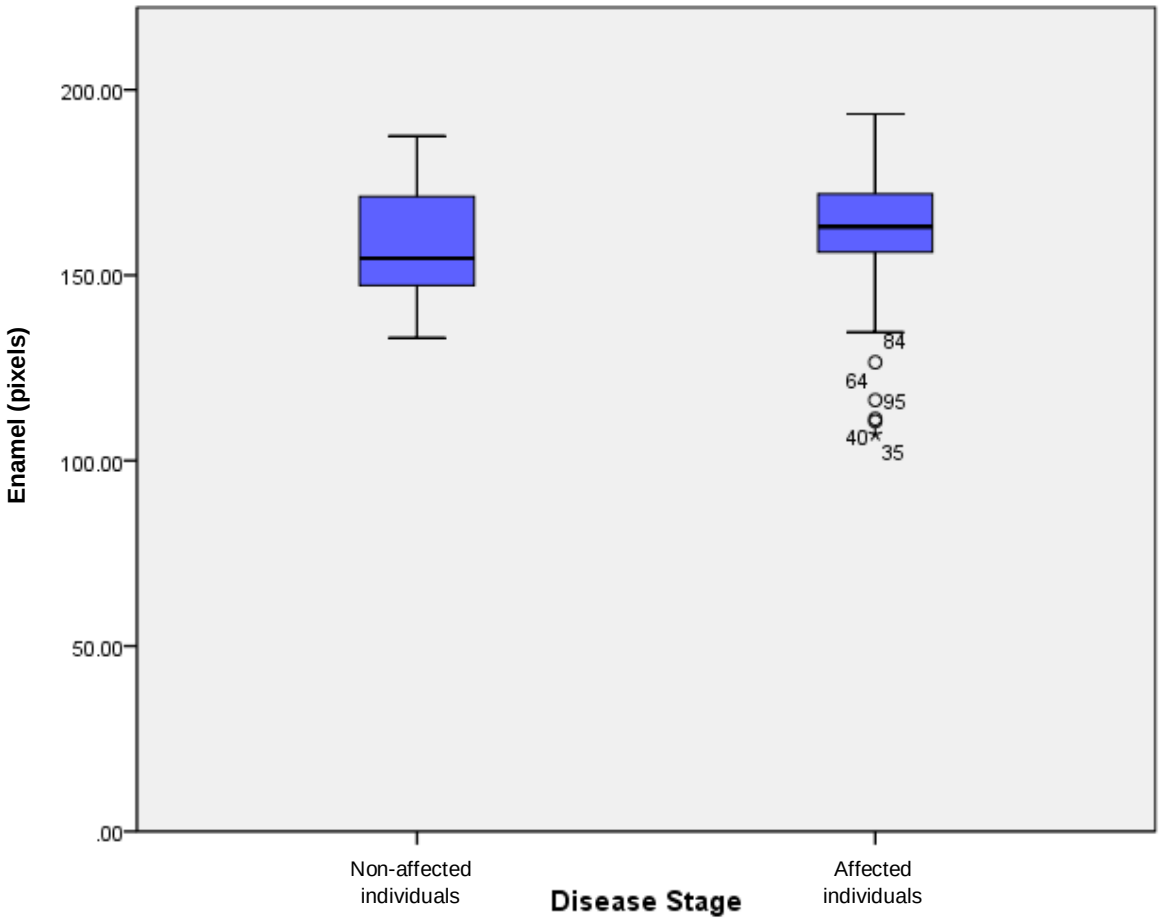
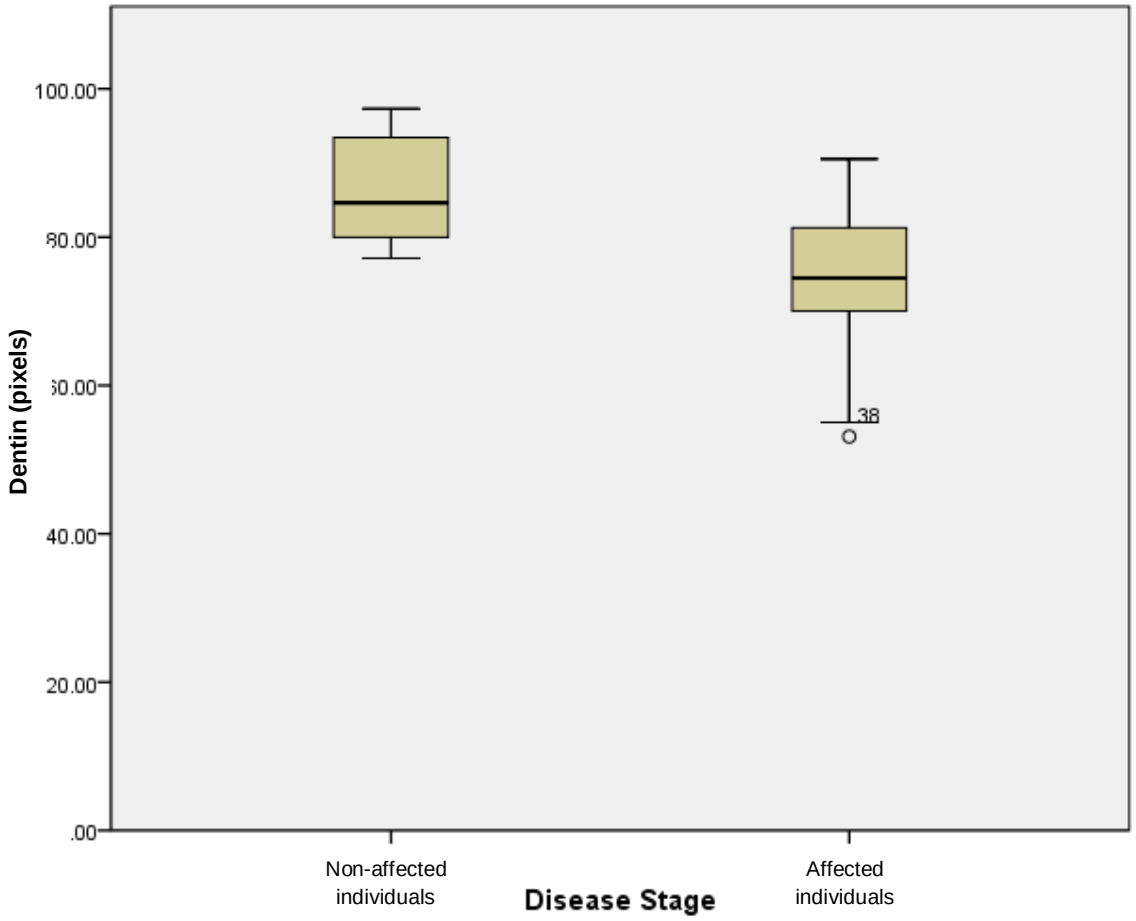


Figure 4: Comparison of dentin gray-scale values between affected and non affected individuals.



3.3 Capítulo 3

SALIVARY PEPTIDE PROFILE OF PATIENTS WITH X-LINKED HYPOPHOSPHATEMIC RICKETS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Abstract

X-linked hypophosphatemic rickets (XLHR) is the most common cause of heritable rickets, with an incidence of 1:20,000 live births, representing more than 80% of familial hypophosphatemic rickets. Saliva is the most easily available and accessible body fluid, which makes it one of the most sought after tools in diagnostic pathology. The present study was performed to evaluate the peptide profile in the saliva of patients with XLHR using high performance liquid chromatography. Unstimulated whole and stimulated parotid saliva were obtained from 8 individuals, both genders, with (AFF) and 8 healthy control individuals, without (CON) XLHR aged from 8 to 66 years. Supernatants were lyophilized and stored at -80^{o}C for later peptide analysis by high performance liquid chromatography, and the salivary flow rate (ml/min) was calculated. The major peak in the HPLC chromatogram of each sample was characterized, both by retention time and absorption spectrum in the 214nm wavelength. Student's t-test and Fischer's exact test were applied for analysis ($p<0.05$). Whole and parotid salivary flows were significantly different ($p = 0.001$), being flow of whole saliva higher (0.518 ± 0.282 mL/min) than parotid saliva (0.124 ± 0.086 mL/min). Whole salivary flow rate was higher in the AFF group (0.698 ± 0.229) than in the CON group (0.339 ± 0.210 mL/min) ($p = 0.006$). Twenty-eight peaks were found in whole and 21 peaks in parotid saliva. Whole saliva of the CON group presented lower number of peaks than AFF group. In parotid saliva, peaks 17 and 28 (retention times: 24 and 39 min) were found exclusively in the AFF group, and peak 13 (retention time: 19 min) exclusively in the CON. Also, no relation between presence/absence of peaks and gender ($p = 1.00$) was found. These results suggest different peptide profile in the parotid and whole saliva of individuals with XLHR. In the present study, these individuals expressed peptides that were not observed by subjects without XLHR, of similar age and gender. Further studies to identify these peptides may assist in the development of future diagnostic tests.

Key words: Hypophosphatemic Rickets, X-Linked Dominant; Peptides; Saliva; Chromatography, High Pressure Liquid.

Introduction

X-linked hypophosphatemic rickets (XLHR) is an X-linked dominant disease first described by Albright (1939). It is the most common cause of heritable rickets, with an incidence of 1:20,000 live births (Davies, Stanbury, 1981), representing more than 80% of familial hypophosphatemic rickets (Jagtap et al., 2012). Key features of XLHR include hypophosphatemia, inappropriately reduced serum 1,25-vitamin D3, defective mineralization and intrinsic osteoblast defects (Bresler et al., 2004), which are clinically characterized by severe bone deformities, especially bowing of the legs, impaired growth, short stature and severe dental deformities (Boukpessi et al., 2010).

The occurrence of the XLHR is due to inactivating mutations in the Phosphate regulating gene with Homologies to Endopeptidase on X chromosome (PHEX), which encodes a zinc-metalloprotease, that cleaves small peptide hormones (Jagtap et al., 2012). It does not seem to cleave fibroblast growth factor 23 (FGF 23) directly, but is involved in the downregulation of FGF 23 by unknown mechanism (Liu et al., 2003). Biochemical evaluation reveals low serum phosphorus, normal calcium, normal or slightly elevated PTH, and decreased TMP/GFR (tubular maximum reabsorption of phosphate per glomerular filtration rate) (Bijvoet et al., 1969). Bone alkaline phosphatase and n-terminal propeptide of type I procollagen were found to be appropriate metabolic bone markers in the assessment of hypophosphatemic rickets / osteomalacia (Nagata et al., 2011).

In the past, serum has been the fluid most often used in disease diagnosis; however saliva has many advantages over both serum and urine. For instance, salivary assays for antibodies, unconjugated steroid hormones and certain drugs are sufficiently sensitive to accurately reflect the blood concentrations of these substances (Slavkin, 1998; Mandel, 1990). Recent advances in saliva´s diagnostic capabilities include salivary detection of HIV-1 (Liu et al., 2011), transcriptome markers KRAS, BMD3L2, ACRV1, and DMP1 in pancreatic cancer (Zhang et al., 2010), identification of MMP-8 and -9 and osteoprotegin, as biomarkers for periodontal disease (Ramseier et al., 2009) and myeloperoxidase biomarkers to detect acute myocardial infarction (Floriano et al., 2009). Because of interest in the link between oral and general health, and due to the noninvasive nature of salivary testing, clinicians are increasingly using salivary analysis to diagnose systemic disease and to monitor general health (Lawrence, 2002; Fonteles et al., 2009; Fonteles et al., 2012; Ribeiro et al., 2013). Salivary levels of various biochemical parameters have been measured in several infectious diseases such as hereditary, autoimmune, endocrine, cancers and psychiatric disorders (Reddy et al., 2012). The composition of saliva includes a large number of protein compounds, which

have been studied by traditional biochemical techniques, including liquid chromatography, gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, mass spectrometry, imunoassays and lectin probe analysis (Chiappin et al., 2007).

High performance liquid chromatography (HPLC) is a form of chromatography developed by Casaba Horváath at Yale University in 1966. It was popularized as a sophisticated improvement over open columns, and it provides more precise and rapid separation (Venkatakrishna et al., 2003). Using an appropriate column it is possible to verify the presence of a great number of peptides; and when HPLC is associated to mass spectrometry these peptides can be identified through their masses (Ribeiro et al., 2013). HPLC has advantages over other biochemical techniques, as it allows for fast and efficient separation and characterization of analytes within a given sample (Reddy et al., 2012).

Although saliva is currently emerging as a diagnostic tool and has been used in the diagnosis of various diseases (Reddy et al., 2012), in XLHR patients, this fluid has not yet been studied. Thus, the present study has evaluated the salivary peptide profile of unaffected or affected patients by X-linked hypophosphatemic rickets using high performance liquid chromatography.

Materials and Methods

Subjects

Study sample consisted of 8 individuals with (AFF) and 8 healthy individuals, from both genders, without (CON) x-linked hypophosphatemic rickets aged from 8 to 66 years (8, 12, 15, 29, 31, 33, 35, 66 years). Affected individuals belonged to 3 generations of the same family and they were matched with the control group by age and gender. The study was approved by the Ethics Committee of the Federal University of Ceará Medical School, Brazil (protocol # 004/11). Consent forms were signed by patients, parents or legal guardians prior to patient enrollment in the study.

Saliva collection

Unstimulated whole and stimulated parotid saliva were obtained. Whole saliva was collected as described by Dawes (1987). In brief, whole saliva was collected by the draining method in which the subject bends the head forward and, after an initial swallow, allows saliva to drip off the lower lip into a graduate container, and spits out at the end of the collection period (Fig. 1A). Parotid saliva was stimulated by applying lemon drops (2-5) onto the lateral border of the tongue and collected from the gland using a Lashley cup (Fig. 1B).

Both salivary collections were performed for 15 min, and after centrifugation at 4°C (Eppendorf Centrifuge 5804 R, Germany), supernatants were lyophilized and stored at -80°C for later peptide analysis. The salivary flow rate (ml/min) was calculated and a protease inhibitor cocktail (Sigma, P2714) was added to samples soon after the collection. Saliva samples were collected 1 h after routine dental brushing and a fasting period of at least 3 h, between 8 and 11:30 am. Patients were instructed to avoid eating, and drinking beverages containing caffeine, or fruit juices throughout the fasting period that preceded saliva sampling or during the saliva collection (Ribeiro et al., 2013).

Peptide analysis

Peptide analysis was performed by high performance liquid chromatography. The samples were re-suspended in 500µL of double-distilled water containing 0.1% trifluoroacetic (TFA) by vortexing for 2 min. Aliquots of 20µL were injected into the SPD-10A HPLC system from Shimadzu. The sample components were separated using a C18 column with a flow of 0.8 mL/min, consisting of two eluents: A (double-distilled water and 0.1% TFA) and B (acetonitrile and 0.1% TFA). Samples were eluted by a gradient of 100% of A from minute 0 to minute 10, followed by ramping to 50% of B from minute 10 to minute 40; and finally 60% of B from minute 40 to minute 50. Equilibration times of 38 min were allowed between injections. Each major peak in the HPLC chromatogram of each sample was characterized both by retention time and absorption spectrum in the 214nm wavelength.

Statistical Analysis

Numerical comparison represented by data on salivary flow rates between AFF and CON groups and between whole and parotid salivas were done by Student's t-test, as data presented a normal distribution. Data was expressed in average ± standard deviation (normal distribution). For comparison between categorical variables (gender, presence/absence of peptides and presence/absence of XLHR) between groups, Fisher's exact test was used. Results were considered statistically significant when $p < 0.05$.

Results

Whole and parotid salivary flows were significantly different ($p = 0.001$). Whole saliva expressed a higher flow rate (0.518 ± 0.282 mL/min) than parotid saliva (0.124 ± 0.086 mL/min). When presence/absence of XLHR was considered, whole salivary flow rate was

higher in the AFF group (0.698 ± 0.229) than in the CON group (0.339 ± 0.210 mL/min) ($p = 0.006$). However, parotid salivary flow rate did not differ between groups (AFF: 0.124 ± 0.102; CON: 0.111 ± 0.073; $p = 0.878$).

Twenty-eight and 21 peaks were respectively identified in whole saliva and parotid saliva. Peaks 5, 12, 14, 15, 16, 22 and 26 (retention times: 6, 16, 20, 21, 22, 30 and 36 min, respectively) were exclusively found in whole saliva and peak 9 (retention time: 13 min) was exclusive for parotid saliva (Fig. 2). No association was observed between presence/absence of peaks and presence/absence of XLHR. However, in the CON group whole saliva presented a lower number of peaks than the AFF group, whereas peaks 6, 8, 14, 15, 16 and 22 (retention times: 10, 12, 20, 21, 22 and 30 min, respectively) were identified exclusively in the AFF group (Fig. 1). In parotid saliva, peaks 17 and 28 (retention times: 24 and 39 min) were only observed in the AFF group, and peak 13 (retention time: 19 min) was only noted in the CON, but these peaks did not associate with the presence of XLHR ($p = 0.077$) (Table 1). No statistical relation was observed between presence/absence of peaks and gender ($p = 1.00$) (Tables 2 and 3).

Discussion

To the best of our knowledge, the present study represents the first attempt to determine salivary peptidome profiling in rickets patients using high performance liquid chromatography. The use of saliva in the present study aimed to add new diagnostic tools for a better comprehension of pathways related to XLHR. Saliva is the most easily available and accessible body fluid, which makes it one of the most sought after tools in diagnostic pathology. Markers expressed in saliva can be used for diagnosis and concurrent patient follow-up of diseases ranging from hereditary disorders to infections, as well as malignances and analysis of therapeutic levels of drugs (Shankar, Dandekar, 2012).

Some peptides have been implicated in the XLHR physiopathology, especially those related to SIBLING (small integrin-binding ligand N-linked glycoprotein) family of proteins, which include osteopontin, bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein (MEPE) (Staines et al., 2012). MEPE expression is markedly elevated in X-linked hypophosphatemic rickets, being circulating levels of MEPE in normal individuals tightly correlated with serum-phosphorus, parathyroid hormone (PTH) and bone mineral density (BMD). In addition, MEPE derived C-terminal ASARM-peptides (acidic-serine-and aspartate-rich motif-peptides) are candidate minhibins and/or phosphatonins. Previously, it was demonstrated that markedly elevated

serum levels of protease-resistant ASARM-peptides occur in mice affected by XLHR, and these peptides accumulate in murine kidneys of these animals (Bresler et al., 2004). In this study, the authors concluded that these peptides are likely responsible for the phosphaturia and defective mineralization in XLHR (Bresler et al., 2004). These phosphorilated peptides which are potent inhibitors of the mineralization are hydrolyzed by the PHEX that encodes a zinc metalloproteinase involved in activation or inactivation of peptides biologically active and their functional absence triggers an aberrant proteolytic process of the matrix proteins which regulate the mineralization (Barros et al., 2013). However, in the present work, peptide profile did not associate with presence/absence of XLHR, suggesting that either ASARM-peptides are not observed in humans, or are solely present in serum thus lacking salivary expression.

In the present study, some characteristics may lead to future studies related the identification of XLHR biomarkers. Presently, the CON group showed a lower number of peaks than the AFF group, being peaks 6, 8, 14, 15, 16 and 22 found exclusively in AFF group. Our research group had previously studied the association between salivary peptide profile and early childhood caries, being collected whole saliva from a group of 106, 10-71 month old children, which was analyzed through liquid chromatography mass spectrometry (LC-MS) (Ribeiro et al., 2013). We found differences in peptide peaks between children with (CE) and without (CF) caries experience, for instance, peaks 13, 14 and 17 were present in both groups, but were higher in the CE group; peaks 2 and 22 were present in the CF group, but virtually absent in the CE group. These results suggested the existence of different peptide profile among children who had and those who had not experienced dental caries (Ribeiro et al., 2013). Similarly, the presence of a different peptide pattern among subjects with and without XLHR can be presently suggested.

Peptide analysis of parotid saliva demonstrated expression of peaks 17 and 28 exclusively in the AFF group, whereas peak 13 was only detected in the CON group, but these differences were not statistically observed. Previous studies also analyzed peptides in parotid saliva. Perinpanayagam et al. (1995) characterized low-molecular weight peptides in parotid saliva and found differences between this study and an earlier work, which could reflect subject variation in processing schemes. Likewise, Ayad et al. (2000) characterized peptides from ductal parotid saliva collected from 9 adults who had remained free from dental caries to address whether peptide composition of human parotid saliva associated with caries experience,. The authors found a significantly higher concentration of peptides corresponding to peaks 14, 15, 16, 17 and 18 in saliva collected from caries-susceptible subjects, and the

concentration of peptides corresponding to peaks 3, 5, 8, 9, 10 and 11 were significantly greater in saliva collected from caries-free subjects.

In summary, the present investigation found a higher number of peaks in whole (28 peaks) than in parotid saliva (21 peaks), being peaks 5, 12, 14, 15, 16, 22 and 26 expressed exclusive in whole saliva, and peak 9 was only observed in parotid saliva. Few studies compared the composition of whole and parotid saliva. Jensen et al. (1992) determined the protein composition of in vitro pellicles, formed from whole saliva and parotid and submandibular secretions by use of synthetic hydroxyapatite as a model for dental enamel. The protein composition of submandibular-derived pellicle was similar to that of parotid-derived pellicle except for the presence of cystatins and the absence of glycosylated proline-rich proteins. In contrast, in vitro pellicle derived from whole saliva presented a vastly different composition consisting primarily of amylase, acidic proline-rich proteins, cystatins, and proteolytically-derived peptides, indicating that acidic phosphoproteins as well as neutral and basic histatins from pure secretions selectively adsorb to hydroxyapatite, whereas in whole saliva some of these proteins are proteolytically degraded, with a dramatically changing on its adsorption pattern. Presently, the greater number of peaks in whole saliva can be justified by protein degradation and less adsorption, which may lead to a higher number of free peptides in whole saliva compared to parotid saliva. In addition, whole saliva presents a higher salivary flow rate compared to parotid saliva; hence, a reduction in salivary flow rates leads to a resultant reduction in total protein concentrations (Matos-Gomes et al., 2010).

In the present work, whole saliva flow rate was higher in the AFF group than in the CON, however parotid flow rate did not differ between groups. To our knowledge, salivary flow rates of individuals with XLHR have not been previously investigated. For instance, percentage contributions of the different salivary glands during unstimulated flow are as follows: 20% from parotid, 65% from submandibular, 7% to 8% from sublingual and less than 10% from numerous minor glands (Edgar, 1990). Thus, the minor contribution of parotid saliva in the unstimulated flow of whole saliva, suggests that flow rate of the parotid glands are not affected by the presence of XLHR, but other salivary glands may have their flow rates affected by this condition. When stimulated, flow rates drastically change percentage contributions from each gland, with the parotid contribution representing over 50% of total salivary secretions (Edgar, 1990). Fluctuations in flow rate in the presence and absence of salivary stimulation may contribute to the absence of difference between stimulated salivary flow rates of parotid saliva between groups.

The present study demonstrated that the peptide profiles of whole and parotid saliva differed in patients with and without XLHR. Further in-depth studies to identify these peptides, may enable future development of salivary diagnostic tests, allowing a cost effective diagnosis of this disease. The use of parotid saliva may render more reproducible results, since in the present study the flow rate of this fluid remained unchanged in the presence of XLHR.

Acknowledgements

We would like to give special thanks Dr. Fábio Sampaio (Federal University of Paraíba) for providing the Lashley cups to parotid collection of saliva.

References

Albright F, Sulkowitch HW, Bloomberg E 1939 A comparison of the effects of vitamin D, dihydrotachysterol (A.T. 10), and parathyroid extraction on the disordered metabolism of rickets. J Clin Invest 18: 165-169.

Ayad M, Van Wuyckhuyse BC, Minaguchi K, Raubertas RF, Bedi GS, Billings RJ, Bowen WH, Tabak LA 2000 The association of basic proline-rich peptides from human parotid gland secretions with caries experience. J Dent Res 79: 976-982.

Barros NM, Hoac B, Neves RL, Addison WN, Assis DM, Murshed M, Carmona AK, McKee MD 2013 Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia. J Bone Miner Res 28: 688-699.

Bijvoet OL, Morgan DB, Fourman P 1969 The assessment of phosphate reabsorption. Clin Chim Acta 26: 15-24.

Boukpessi T, Gaucher C, Léger T, Salmon B, Le Faouder J, Willig C, Rowe PS, Garabédian M, Meihac O, Chaussain C 2010 Abnormal presence of the matrix extracellular phosphoglycoprotein-derived acidic serine-and aspartate-richmotif peptide in human hypophosphatemic dentin. Am J Pathol 177: 803-812.

Bresler D, Bruder J, Mohnike K, Fraser WD, Rowe PS 2004 Serum MEPE-ASARM-peptides are elevated in X-linked rickets (HYP): implications for phosphaturia and rickets. J Endocrinol 183: R1-R9.

Chiappin S, Antonelli G, Gatti R, De Palo EF 2007 Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta 383: 30-40.

Davies M, Stanbury S 1981 The rheumatic manifestations of metabolic bone disease. Clin Rheum Dis 7: 595-646.

Dawes C 1987 Physiological factors affecting salivar flow rate, oral sugar clearance, and the sensation of dry mouth in man. J Dent Res 66: 648-653.

Edgar WM 1990 Clinical implications of saliva: report of a consensus meeting. Br Dent J 169: 96-98.

Fonteles CS, Dos Santos CF, da Silva Alves KS, de Miranda Mota AC, Damasceno JX, Fonteles MC 2012 Comparative proteomic analysis of human whole saliva of children with protein-energy undernutrition. Nutrition 28: 744-748.

Fonteles CSR, Guerra MH, Ribeiro TR, Mendonça DN, de Carvalho CB, Monteiro AJ, Toyama DO, Toyama MH, Fonteles MC 2009 Association of free amino acids with caries experience and mutans streptococci levels in whole saliva of children with early childhood caries. Arch Oral Biol 54: 80-85.

Floriano PN, Christodoulides N, Miller CS, Ebersole JL, Spertus J, Rose BG, Kinane DF, Nobak MJ, Steinhubl S, Acosta S, Mohanty S, Dharshan P, Yeh CK, Redding S, Furmaga W, McDevitt JT 2009 Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: a feasible study. Clin Chem 55: 1530-1538.

Jagtap VS, Sarathi V, Lila AR, Bandgar T, Menon P, Shah NS 2012 Hypophosphatemic rickets. Indian J Endocrinol Metab 16: 177-182.

Jensen JL, Lamkin MS, Oppenheim FG 1992 Adsorption of human salivary proteins to hydroxyapatite: a comparison between whole saliva and glandular salivary secretions. J Dent Res 71: 1569-1576.

Lawrence HP 2002 Salivary markers of systemic disease: noninvasive diagnosis of disease and monitoring of general health. J Can Dent Assoc 68: 170-174.

Liu C, Geva E, Mauk M, Qiu X, Abrams WR, Malamud D, Curtis K, Owen SM, Bau HH 2011 Na isothermal amplification reactor with an integrated isolation membrane for point-of-care detection of infectious diseases. Analyst 136: 2069-2076.

Liu S, Guo R, Simpson LG, Xiao ZS, Burnham CE, Quarles LD 2003 Regulation of fibroblastic growth fator 23 expression but not degradation by PHEX. J Biol Chem 278: 37419-37426.

Matos-Gomes N, Katsurayama M, Makimoto FH, Santana LL, Paredes-Garcia E, Becker MA, Dos-Santos MC 2010 Psychological stress and its influence on salivar flow rate, total protein concentration and IgA, IgG and IgM titers. Neuroimmunomodulation 17: 396-404.

Mandel ID 1990 The diagnostic uses of saliva. J Oral Pathol Med 19: 119-125.

Nagata Y, Imanischi Y, Ishii A, Kurajoh M, Motoyama K, Morioka T, Naka H, Mori K, Miki T, Emoto M, Inaba M 2011 Evaluation of bone markers in hypophosphatemic rickets/osteomalacia. Endocrine 40: 315-317.

Perinpanayagam HE, Van Wuyckhuyse BC, Ji ZS, Tabak LA 1995 Characterization of low-molecular-weight peptides in human parotid saliva. J Dent Res 74: 345-350.

Ramseier CA, Kinney JS, Herr AE, Braun T, Sigai JV, Shelburne CA, Rayburn LA, Tran HM, Singh AK, Giannobile WV 2009 Identification of pathogen and host-response markers correlated with periodontal disease. J Periodontol 80: 436-446.

Reddy I, Sherlin HJ, Ramani P, Premkumar P 2012 Amino acid profile of saliva from patients with oral squamous cell carcinoma using high performance liquid chromatography. J Oral Sci 54: 279-283.

Ribeiro TR, Dria KJ, de Carvalho CB, Monteiro AJ, Fonteles MC, de Moraes Carvalho K, Fonteles CS 2013 Salivary peptide profile and its association with early childhood caries. Int J Paediatr Dent 23: 225-234.

Shankar AA, Dandekar RC 2012 Salivary biomarkers – an update. Dent Update 39: 571-572.

Slavkin HC 1998 Toward molecular based diagnostics for the oral cavity. J Am Dent Assoc 129: 1138-1143.

Staines KA, MacRae VE, Farquharson C 2012 The importance of the SIBLING family of proteins on skeletal mineralization and bone remodeling. J Endocrinol 214: 241-255.

Venkataksisha K, Kartha VB, Pai KM, Krishna CM, Ravikiran O, Kurian J, Alexander M, Ullas G 2003 HPLC-LIF for early detection of oral cancer. Curr Sci 84: 551-557.

Zhang L, Farrell JJ, Zhou H, Elashoff D, Akin D, Park NH, Chia D, Wong DT 2010 Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. Gastroenterology 138: 949-957.

Table 1: Presence of peaks in whole and parotid saliva in the affected and control groups.

	Whole Saliva		Parotid Saliva	
Peaks	**AFF**	**CON**	**AFF**	**CON**
1	8	8	8	8
2	0	1	5	3
3	6	5	4	3
4	0	3	8	8
5	8	5	0	0
6	1	0	2	2
7	7	8	6	6
8	1	0	2	4
9	0	0	3	6
10	1	1	5	3
11	0	3	8	8
12	5	4	0	0
13	3	0	0	3
14	1	0	0	0
15	1	0	0	0
16	3	0	0	0
17	3	5	4	0
18	7	6	5	2
19	1	3	4	1
20	3	4	1	5
21	5	5	6	5
22	2	0	0	0
23	5	4	1	1
24	2	5	4	6
25	1	3	1	1
26	1	0	0	0
27	2	3	1	1
28	3	3	4	0

Table 2: Relation between presence/absence of peaks and gender in WHOLE SALIVA.

	Presence			Absence		
Peaks	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**
1	8	8	16	0	0	0
2	1	0	1	7	8	15
3	6	5	11	2	3	5
4	2	1	3	6	7	13
5	6	7	13	2	1	3
6	0	1	1	8	7	15
7	8	7	15	0	1	1
8	0	1	1	8	7	15
9	0	0	0	8	8	16
10	1	1	2	7	7	14
11	1	2	3	7	6	13
12	4	5	9	4	3	7
13	1	2	3	7	6	13
14	0	1	1	8	7	15
15	0	1	1	8	7	15
16	1	2	3	7	6	13
17	4	4	8	4	4	8
18	7	6	13	1	2	3
19	1	3	4	7	5	12
20	4	3	7	4	5	9
21	6	4	10	2	4	6
22	1	1	2	7	7	14
23	6	3	9	2	5	7
24	3	4	7	5	4	9
25	3	1	4	5	7	12
26	0	1	1	8	7	15
27	4	1	5	4	7	11
28	3	3	6	5	5	10
Total	81	78	159	143	146	289

Table 3: Relation between presence/absence of peaks and gender in PAROTID SALIVA.

	Presence			Absence		
Peaks	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**
1	8	8	16	0	0	0
2	3	5	8	5	3	8
3	3	4	7	5	4	9
4	8	8	16	0	0	0
5	0	0	0	8	8	16
6	1	3	4	7	5	12
7	7	5	12	1	3	4
8	3	3	6	5	5	10
9	4	5	9	4	3	7
10	4	4	8	4	4	8
11	8	8	16	0	0	0
12	0	0	0	8	8	16
13	1	2	3	7	6	13
14	0	0	0	8	8	16
15	0	0	0	8	8	16
16	0	0	0	8	8	16
17	2	2	4	6	6	12
18	3	4	7	5	4	9
19	3	2	5	5	6	11
20	3	3	6	5	5	10
21	6	5	11	2	3	5
22	0	0	0	8	8	16
23	1	1	2	7	7	14
24	7	3	10	1	5	6
25	1	1	2	7	7	14
26	0	0	0	8	8	16
27	1	1	2	7	7	14
28	2	2	4	6	6	12
Total	79	79	158	145	145	290

Figure 1: (A) Whole saliva collection. (B) Parotid saliva collection with Lasley cup placed in the parotid duct.

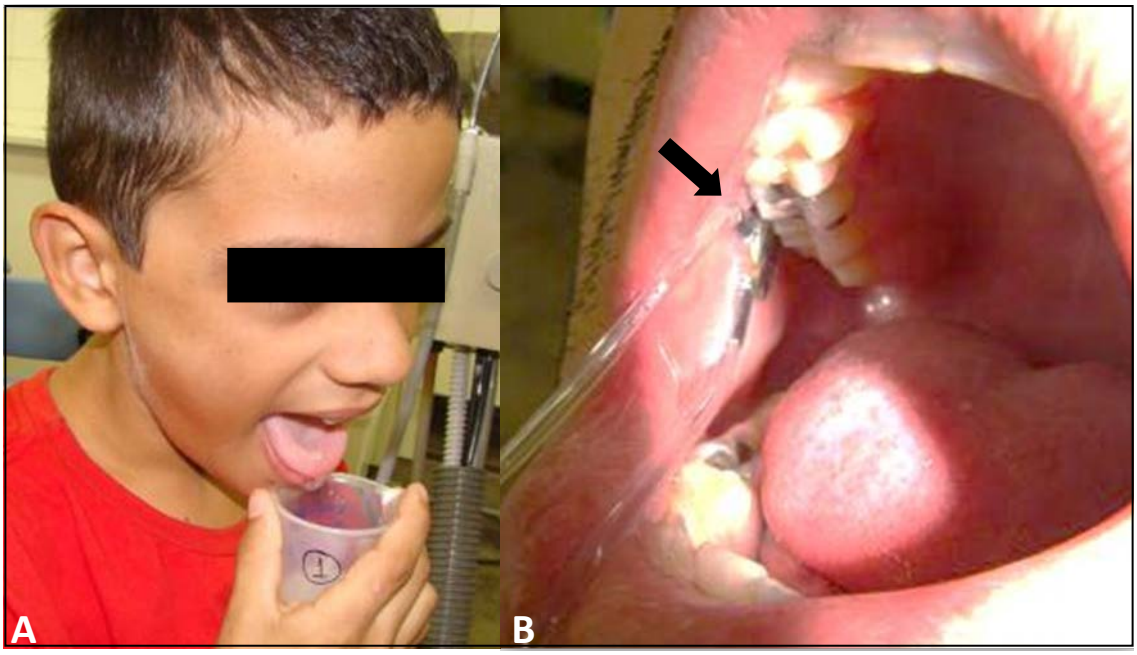
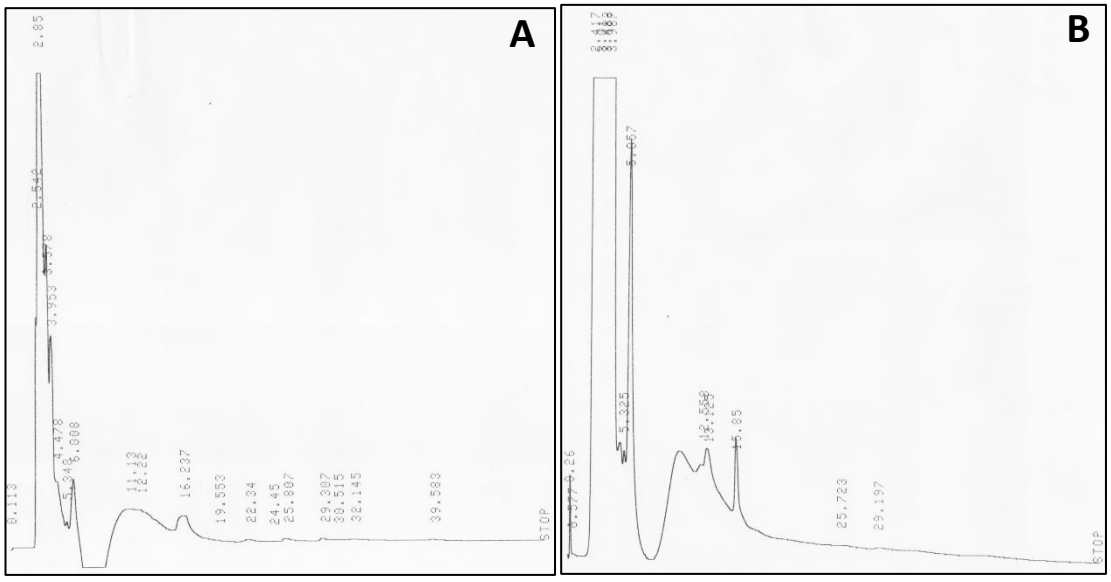


Figure 2: Chromatograms of whole (A) and parotid (B) saliva.



3.4 Capítulo 4

COMPARISON BETWEEN WHOLE AND PAROTID SALIVARY PROTEIN PROFILE IN A FAMILY WITH X-LINKED HYPOPHOSPHATEMIC RICKETS

Abstract

X-linked hypophosphatemic rickets (XLHR) is a dominant disease characterized by renal phosphate wasting and abnormal vitamin D metabolism, being PHEX the gene defective in this disorder. This study was performed in order to characterize salivary proteins in group of individuals affected by XLHR using unidimensional electrophoresis. Eight individuals affected with (AFF) and 8 healthy individuals, both genders, without (CON) XLHR aged from 8 to 66 years participated in this study. Unstimulated whole and stimulated parotid saliva were obtained. Both salivary collections were performed for 15 min, and after centrifugation at 4^{o}C supernatants were lyophilized and stored at -80^{o}C for later protein analysis. Total protein concentration was determined by the Bicinchoninic Acid Protein (BCA) method, being all samples analyzed in triplicate. Proteins were characterized according to their molecular weights within the unidimensional electrophoresis. After image acquisition and statistical analysis, results were obtained. Total protein concentration was different between whole and parotid saliva ($p < 0.001$), being higher concentration found in whole saliva (102.603 ± 42.336 µg/mL) than in parotid saliva (0.699 ± 0.438 µg/mL). Unidimensional electrophoresis showed 12 bands in whole saliva from both groups while 13 bands were present in parotid saliva. Band with 60 kDa was present exclusively in parotid saliva. Bands with 102 kDa, 48 kDa and 24 kDa presented higher intensity in whole saliva of CON group ($p = 0.015$, $p = 0.043$ and $p = 0.022$). In conclusion, the differences found in protein profile of AFF group compared to CON group, including differences in bands intensity, may lead to biomarkers of XLHR in saliva. Further studies are necessary to identify these proteins and compare salivary and serum concentrations to confirm our findings.

Key words: X-linked hypophosphatemic rickets; proteins, saliva, electrophoresis

Introduction

The final structure of most proteins and peptides present in whole saliva is defined by a complex series of molecular processes. Thus, knowledge on the composition of saliva in terms of proteins and peptides is important, not just to determine function of these components, but also due to the growing interest in saliva-based diagnostics (Hermerhorst and Oppenheim, 2007). The use of saliva has provided a substantial addition to the diagnostic armamentarium as an investigative tool for disease processes and disorders (Chiappin et al., 2007). In addition, the use of this oral fluid has many advantages, including the simple and non-invasive method of collection and its easy, low-cost storage (Lima et al., 2010).

X-linked hypophosphatemic rickets (XLHR) is a dominant disease characterized by renal phosphate wasting and abnormal vitamin D metabolism, being PHEX the gene defective in this disorder (Holm et al., 1997). Clinically, this condition is characterized by growth failure and bowing of the legs, being usually treated with phosphate and a vitamin D preparations (Verge et al., 1991). One of the most important oral alterations elicited by this condition is the recurrent formation of spontaneous abscesses (Hernández and Laguna, 2013). Bone mineralization is possible via complex interactions among fibroblast growth factor 23 (FGF23), phosphate-regulating gene with homologies to endopeptidases in the X-chromosome (PHEX), and matrix extracellular phosphoglycoprotein, being a loss-of-function mutation in PHEX responsible for the disruption of this interaction leading to hypophosphatemic rickets (Murthy, 2009).

Proteins can be detected by a number of methodologies. Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, has proven highly useful for resolving and characterizing protein components of mixtures in both unidimensional and two-dimensional systems (Shapiro et al., 1967; O'Farrell, 1975). Considering the protein interactions in XLHR and based in the fact that saliva was not previous studied in these patients, the present study was performed to characterize salivary protein profile in this condition using unidimensional electrophoresis.

Materials and Methods

Subjects

Eight individuals with (AFF) and 8 healthy individuals without (CON) x-linked hypophosphatemic rickets, both genders, aged from 8 to 66 years (8, 12, 15, 29, 31, 33, 35, 66 years) participated in this study. Affected individuals belonged to 3 generations of the same family and they were matched with the control group by age and gender. The study was

approved by the Ethics Committee of the Federal University of Ceará Medical School, Brazil (protocol # 004/11). Consent forms were signed by patients, parents or legal guardians prior to patient enrolment in the study.

Saliva collection

Before saliva collection an oral clinic exam was performed to evaluate the general condition of the oral cavity and the number of teeth. Unstimulated whole and stimulated parotid saliva were obtained. Whole saliva was collected as described by Dawes (1987). In brief, whole saliva was collected by the draining method in which the subject bends the head forward and, after an initial swallow, allows saliva to drip off the lower lip into a graduate container, and at the end of the collection period, the subject spit out. While parotid saliva was stimulated by applying lemon drops (2-5) onto the lateral border of the tongue, and collected from the gland using a Lashley cup. Both salivary collections were performed for 15 min, and after centrifugation at 4^{o}C, 12 000G, for 10 min (Eppendorf Centrifuge 5804 R, Germany), supernatants were lyophilized and stored at -80^{o}C for posterior analysis. Protease inhibitor cocktail (Sigma, P2714) was added to samples soon after the collection. Saliva samples were collected 1 h after routine dental brushing and a fasting period of at least 3 h, between 8 and 11:30 am. Subjects were instructed to avoid eating, and drinking beverages containing caffeine, or fruit juices throughout the fasting period that preceded saliva sampling or during the saliva collection (Ribeiro et al., 2013).

Determination of total protein concentration

The lyophilized samples were reconstituted by adding 1mL of Milli Q water (Milli-Q Plus Ultrapure water system, Millipore Corp., MA, USA) and homogenized with a mechanical agitator (Vortex AP-56, Phoenix, São Paulo, Brazil). Total protein concentration was determined by the Bicinchoninic Acid Protein (BCA) method using BCA assay kit (BCA-1, Sigma-Aldrich). All samples were analyzed in triplicate. The BCA working reagent was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B until the color of the solution became light green. Two 96 well plate assay (plate 1: whole saliva, plate 2: parotid saliva) were prepared with a blank, BSA protein standards and saliva samples; and incubated at 50^{o}C. The absorbance at 620nm was recorded and the protein concentration was determined by comparison to a standard curve.

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

All reconstituted samples (whole and parotid saliva) were briefly vortexed and 50µL aliquots were loaded on 2 polyacrylamide gels (12 x 14 cm, 12.5%), being one for whole saliva and the other one for parotid saliva. Proteins were separated according to their molecular weight within the electrophoresis apparatus (IPGphor and Hoefer SE600; Amersham Pharmacia Biotech, Cambridge, UK). At the end of the running session, the gel was fixed for 30 min in 10% trichloroacetic acid, stained with Coomassie blue for 1 h, and distained overnight with distaining solution containing 5% acetic acid, 10% methanol and H_2O (2550 mL).

Image Acquisition and Analysis

The gels were digitalized (FX; Bio-Rad, Hercules; CA, USA), and the images were processed with ImageJ 1.43u software (Wayne Rasband, National Institutes of Health, USA). Molecular weights of the proteins were estimated based on comparisons to prestained broad-range protein standards (Bio-Rad). Numerical comparison (bands intensity and total proteins dosage) between AFF and CON groups and between whole and parotid saliva were performed using Student's t-test when data presented normal distribution and Mann-Whitney test when data did not present normal distribution. Data were expressed in mean ± standard deviation (normal distribution) or median with percentiles 25 and 75 (abnormal distribution). Results were considered statistically significant when $p < 0.05$. Statistical analysis were performed with Sigmaplot 9.0 program

Results

Healthy patients presented a greater number of teeth (mean = 26), while AFF patients presented a mean of 3.5 teeth, being this difference statistically significant ($p = 0.039$).

Total protein concentrations differed in whole and parotid saliva ($p < 0.001$), being higher concentrations found in whole saliva (102.603 ± 42.336 µg/mL) than in parotid saliva (0.699 ± 0.438 µg/mL). Considering whole and parotid saliva independently, differences between AFF and CON groups were not found (whole saliva: 104.270 ± 42.738 µg/mL (AFF), 100.937 ± 44.808 (CON), $p = 0.881$; parotid saliva: 0.695 ± 0.519 µg/mL (AFF), 0.704 ± 0.377 µg/mL (CON), $p = 0.970$).

Unidimensional electrophoresis showed 12 bands in whole saliva from both groups, while 13 bands were present in parotid saliva (3, 4, 6, 12, 24, 30, 48, 60, 81, 89, 102,

120 and 158 kDa). Band with 60 kDa was present exclusively in parotid saliva. All the other bands were present in both types of saliva. Protein profiles presented through electrophoresis differed in the intensity of protein bands (Fig. 1). Bands with 102 kDa, 48 kDa and 24 kDa presented higher intensity in whole saliva of the CON group ($p = 0.015$, $p = 0.043$ and $p = 0.022$) (Fig. 2). Comparison of all bands between AFF and CON within parotid saliva showed no significant differences ($p > 0.05$).

Discussion

The interest in saliva has increased in the last few years for its potential to diagnose viral, bacterial and systemic diseases (Schipper et al., 2007). This fluid is a promising option for diagnosing certain disorders and monitoring the evolution of certain pathologies or the dosage of medicines or drugs; and the advantages of saliva as a diagnostic tool include the facility to obtain and the positive correlation between many parameters in serum and saliva (Llena-Puy, 2006). Because of this increasing importance of saliva as a diagnostic tool, this preliminary work was performed in order to study salivary proteins through their molecular masses using unidimensional electrophoresis in patients with X-linked hypophosphatemic rickets.

To our knowledge, there are no previous studies on salivary research in rickets. However, total protein concentration in saliva have been studied in other conditions such as Prader-Willi syndrome (Saeves et al, 2012), oral squamous cell carcinoma (Fuchs et al., 2011), protein energy undernutrition (Fonteles et al., 2012) overweight and obesity (Pannunzio et al., 2010) and Fanconi anemia (Mattioli et al., 2010). In the present study, considering whole and parotid saliva, we did not find differences between total protein concentrations in whole and parotid saliva when comparing AFF and CON groups. Other studies have described similar results when investigating salivary mucin and total protein levels from submandibular glands in patients before and after radiotherapy for head-and-neck cancer (Dijkema et al., 2012). In contrast, Pannunzio et al. (2010) performed a study to determine if stimulated whole saliva parameters were influenced by an increase in Body Mass Index, and found an increase in the concentrations of protein in the obese group compared to the overweight and control groups. In addition, Henskens et al. (1996) when studying cystatins in healthy and periodontitis subjects using parotid saliva also found differences in total protein levels, which were significantly higher in the saliva of patients with periodontitis.

We also analyzed the number of teeth of patients from the 2 studied groups. Healthy patients presented a greater number of teeth than AFF group. This finding is

consistent with the nature of XLHR, because in this condition an atypical premature spontaneous loss of teeth is observed due to periodontal defects (Ye et al., 2011; Al-Jundi et al., 2011). A study tried to establish an association between number of teeth and salivary factors, including total protein concentration however, no significant association was found when considering total proteins (Masamura et al., 1995). So the number of teeth did not influence total protein concentration in saliva (Masamura et al., 1995), which is in aggreement with our data, where despite the difference in the number of teeth between groups, total protein concentration did not differ. Whole saliva presented higher levels of total protein concentrations than parotid saliva. Whole saliva concentrations originate from sources other than the parotid gland (Henskens et al., 1996), explaining our findings. Also, whole saliva is a complex mix of fluids from major (parotid, submandibular and sublingual glands) and minor salivary glands (found in the lower lip, tongue, palate, cheeks and pharynx), and from gingival crevicular fluid, which contains oral bacteria and food debris (Edgar, 1992; Roth et al., 1981).

Unidimensional electrophoresis allowed the detection of differences in the expression of protein bands between whole and parotid saliva, being 12 bands found in whole and 13 bands found in parotid saliva. The 60kDa band was present exclusively in parotid saliva. This greater number of bands in parotid saliva probably occurred due to the fact that different sources are responsible for the whole saliva composition (Henskens et al., 1996), so different interactions may occur which leads to these differences. The difference between parotid and whole saliva may reflect constitutive secretion of all proteins at low levels of stimulation (Rudney et al., 1991). A 60kDa band was found in parotid saliva, some protein types express this molecular mass, such as the splicing factor 3A subunit 3 (Magdaleno et al., 2006; Mckee et al., 2005). Further studies are needed to identify this protein band in human parotid saliva. In the present study, protein profiles presented through electrophoresis differed in the expression of protein bands intensity, in which bands with 102kDa, 48kDa and 24kDa presented higher intensity in whole saliva of the CON group; however, in the parotid saliva, these differences were not found. As the intensity of staining and “thickness” of protein bands are indicative of their relative abundance (Sasse and Gallagher, 2004), these proteins were more abundant in the CON group. The lower concentration of these proteins in the AFF group could be associated with the occurrence of XLHR. Further studies investigating specific protein-related XLHR are necessary, such as non-collagenous proteins. Among these proteins, there is the small integrin-binding ligand N-linked glycoprotein (SIBLING) family. This group of proteins is represented by osteopontin (OPN), bone sialoprotein (BSP), dentin matrix

protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE) (Staines et al., 2012; Suzuki et al., 2012; Barros et al., 2013; Salmon et al., 2013)

In conclusion, in the present study healthy subjects and individuals with hyphosphatemic rickets expressed the same protein bands, as well as levels of total protein concentrations. Nevertheless, protein profile of patients with X-linked hypophastemic rickets, differed from patients without the disease by expressing lower abundance of specific protein bands. Future studies should focus in identifying these proteins, and evaluating salivary and serum concentrations of these molecules. .

References

Al-Jundi SH, Hammad MM, Dabous I (2011). Case report: hypophosphatemic rickets and aggressive periodontitis: a review of the role of dentine matrix protein 1 in the pathogenesis. *Eur Arch Paediatr Dent* 12: 46-50.

Barros NM, Hoac B, Neves RL, Addison WN, Assis DM, Murshed M, Carmona AK, McKee MD (2013). Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia. *J Bone Miner Res* 28: 688-699.

Chiappin S, Antonelli G, Gatti R, De Palo EF (2007). Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta* 383: 30-40.

Dawes C **(**1987). Physiological factors affecting salivar flow rate, oral sugar clearance, and the sensation of dry mouth in man. *J Dent Res* 66: 648-653.

Dijkema T, Terhaard CH, Roesink JM, Raaijmakers CP, van den Keijbus PA, Brand HS, Veerman EC (2012). MUC5B levels in submandibular gland saliva of patients treated with radiotheraphy for head-and-neck cancer: a pilot study. *Radiat Oncol* 7:91.

Edgar WM (1992). Saliva: its secretion, composition and functions. *Br Dent J* 172: 305-312.

Fonteles CS, Dos Santos CF, da Silva Alves KS, de Miranda Mota AC, Damasceno JX, Fonteles MC (2012) Comparative proteomic analysis of human whole saliva of children with protein-energy undernutrition. *Nutrition* 28: 744-748.

Fuchs PN, Rogic D, Vidovic-Juras D, Susic M, Milenovic A, Brailo V, Boras VV (2011). Salivary analytes in patients with oral squamous cell carcinoma. *Coll Antropol* 35: 359-362.

Henskens YM, van den Keijbus PA, Veerman EC, Van der Weijden GA, Timmerman MF, Snoek CM, Van der Velden U, Nieuw Amerongen AV (1996). Protein composition of whole and parotid saliva in healthy and periodontitis subjects. Determination of cystatins, albumin, amylase and IgA. *J Periodontal Res* 31: 57-65.

Helmerhorst EJ, Oppenheim FG (2007). Saliva: a dynamic proteome. *J Dent Res* 86: 680-693.

Hernandéz GG, Laguna FB (2013). Dental characteristics of hypophosphatemic rickets. Case report. *Rev Odont Mex* 17: 101-108.

Holm IA, Huang X, Kunkel LM (1997). Mutational analysis of the PEX gene in patients with X-linked hypophosphatemic rickets. *Am J Hum Genet* 60: 790-797.

Lima DP, Diniz DG, Moimaz SA, Sumida DH, Okamoto AC (2010). Saliva: reflection of the body. *Int J Infect Dis* 14: 184-188.

Llena-Puy C (2006). The role of saliva in maintaining oral health and as an aid to diagnosis. *Med Oral Patol Oral Cir Bucal* 11: E449-E455.

Magdaleno S, Jensen P, Brumwell CL, Seal A, Lehman K, Asbury A, Cheung T, Cornelius T, Batten DM, Eden C, Norland SM, Rice DS, Dosooye N, Shakya S, Mehta P, Curran T (2006). BGEM: an in situ hybridization database of gene expression in the embryonic and adult mouse nervous system. *PLoS Biol* 4: e86.

Masamura K, Inaba R, Iwata H (1995). Salivary calcium and total protein in relation to dental caries. *Nihon Eiseigaku Zasshi* 50: 886-892.

Mattioli TM, Koubik AC, de Oliveira Ribas M, França BH, Brancher JA, de Lima AA (2010). Salivary flow rate, calcium, urea, total protein, and amylase levels in fanconi anemia. *J Pediatr Hematol Oncol* 32: e46-e49.

McKee AE, Minet E, Stern C, Riahi S, Stiles CD, Silver PA (2005). A genome-wide in situ hybridization map of RNA-binding proteins reveals anatomically restricted expression in the developing mouse brain. *BMC Dev Biol* 20:14.

Murthy AS (2009). X-linked hypophosphatemic rickets and craniosynostosis. *J Craniofac Surg* 20: 439-442.

O'Farrell PH (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250: 4007-4021.

Pannunzio E, Amancio OM, Vitalle MS, Souza DN, Mendes FM, Nicolau J (2010). Analysis of the stimulated whole saliva in overweight and obese school children. *Rev Assoc Med Bras* 56: 32-36.

Ribeiro TR, Dria KJ, de Carvalho CB, Monteiro AJ, Fonteles MC, de Moraes Carvalho K, Fonteles CS (2013). Salivary peptide profile and its association with early childhood caries. *Int J Paediatr Dent* 23: 225-234.

Roth G, Calmes R, editors. Salivary glands and saliva. In: Oral biology. St Louis: CV Mosby; 1981: 196-236.

Rudney JD, Krig MA, Neuvar EK, Soberay AH, Iverson L (1991). Antimicrobial proteins in human unstimulated whole saliva in relation to each other, and to measures of health status, dental plaque accumulation and composition. *Arch Oral Biol* 36: 497-506.

Saeves R, Reseland JE, Kvam BM, Sandvik L, Nordgarden H (2012). Saliva in Prader-Willi syndrome: quantitative and qualitative characteristics. *Arch Oral Biol* 57: 1335-1341.

Salmon B, Bardet C, Khaddam M, Naji J, Coyac BR, Baroukh B, Letourneur F, Lesieur J, Decup F, Le Denmat D, Nicoletti A, Pollard A, Rowe PS, Huet E, Vital SO, Linglart A, McKee MD, Chaussain C (2013) MEPE-derived ASARM peptide inhibits odontogenic differentiation of dental pulp stem cells and impairs mineralization in tooth models of X-linked hypophosphatemia. *Plos one* 8: e56749.

Sasse J, Gallagher SR. Staining proteins in gels. Curr Protoc Immunol (2004) Feb; Chapter 8: Unit 8.9.

Schipper RG, Silletti E, Vingerhoeds MH (2007). Saliva as research material: biochemical, physicochemical and practical aspects. *Arch Oral Biol* 52: 1114-1135.

Shapiro AL, Vinuela E, Maizel JV Jr (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem Biophys Res Commun* 28: 815-820.

Staines KA, MacRae VE, Farquharson C (2012) The importance of the SIBLING family of proteins on skeletal mineralization and bone remodeling. *J Endocrinol* 214: 241-255.

Suzuki S, Haruyama N, Nishimura F, Kulkarni AB (2012) Dentin sialophosphoprotein and dentin matrix protein-1: two highly phosphorylated proteins in mineralized tissues. *Arch Oral Biol* 57: 1165-1175.

Verge CF, Lam A, Simpson JM, Cowell CT, Howard NJ Silink M (1991). Effects of therapy in X-linked hypophosphatemic rickets. *N Engl J Med* 325: 1843-1848.

Ye L, Liu R, White N, Alon US, Cobb CM (2011). Periodontal status of patients with hypophosphatemic rickets: a case series. *J Periodontol* 82: 1530-1535.

Figures:

Figure 1: Unidimensional electrophoresis gels representing protein bands found in whole and parotid saliva. (A) Whole saliva from affected group. (B) Whole saliva from control group. Bands with 102 kDa, 48 kDa and 24 kDa had higher intensities when compared to affected group in whole saliva. (C) Parotid saliva from affected group. (D) Parotid saliva from control group. 60kDa band was present exclusively in parotid saliva.

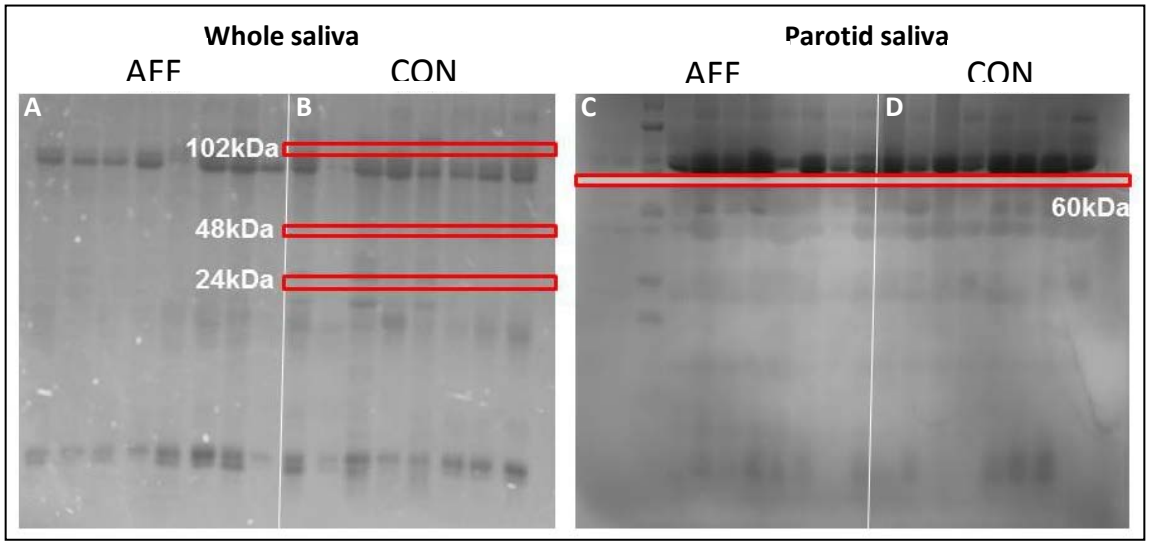
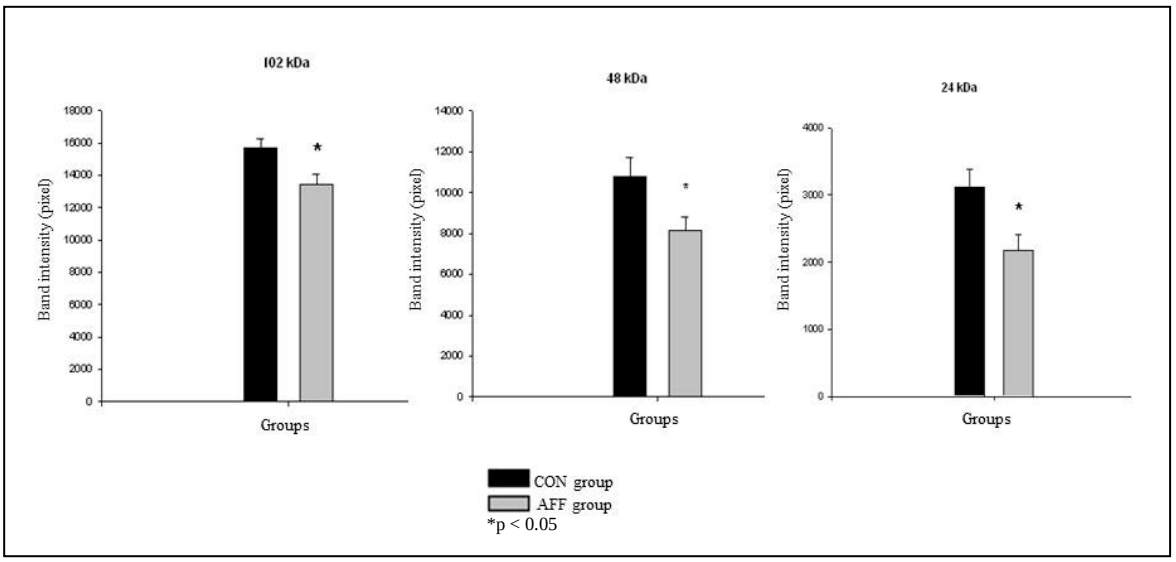


Figure 2: Bands which presented different intensities in parotid saliva between CON and AFF groups.



4 CONCLUSÕES GERAIS

A partir dos resultados obtidos pôde-se concluir que:

1. Os achados orais do Raquitismo Hipofosfatêmico Familiar associados às manifestações sistêmicas dessa doença foram importantes para o correto diagnóstico e estabelecimento de tratamento odontológico adequado, que foi selecionado de acordo com as idades e especificações de cada caso.

2. A microtomografia computadorizada permitiu a análise de imagens com resolução bem superior às radiografias convencionais, podendo ser observado um diferente padrão de mineralização da dentina em pacientes afetados pela doença em estudo, quando comparado à dentina de pacientes não afetados. Em relação ao padrão de mineralização do esmalte, não houve diferença entre os grupos.

3. Diferentes padrões de peptídeos e proteínas em saliva total e de parótida foram encontrados em indivíduos afetados comparando-se com indivíduos saudáveis. Nenhuma associação foi encontrada em relação à presença/ausência de picos e presença/ausência de XLHR.

4. Considerando as proteínas, a concentração de proteínas totais foi maior na saliva total. No entanto, analisando os tipos salivares em separado, não houve diferença entre os grupos estudados. A eletroforese unidimensional demonstrou 12 bandas em saliva total e 13 bandas em saliva de parótida, sendo a banda com 60kDa exclusiva da saliva de parótida. Na saliva total, as bandas com 102kDa, 48kDa e 24kDa apresentaram maior intensidade no grupo controle.

REFERÊNCIAS

ARICETA, G.; LANGMAN, C. B. Growth in x-linked hypophosphatemic rickets. **Eur. J. Pediatr.**, v. 166, p. 303-309, 2007.

BARONCELLI, G. I.; ANGIOLINI, M.; NINNI, E.; GALLI, V.; SAGGESE, R.; GIUCA , M. R. Prevalence and pathogenesis of dental and periodontal lesions in children with x-linked hypophosphatemic rickets. **Eur. J. Paediatr. Dent**., v. 7, p. 61-66, 2006.

BATRA, P.; TEJANI, Z.; MARS, M. X-Linked hypophosphatemia dental and histologic findings. **J. Can. Dent. Assoc.,** v. 72, p. 69-72, 2006.

BELAZI, M. A.; GALLI-TSINOPOULOU, A.; DRAKOULAKOS, D.; FLEVA, A.; PAPANAYIOTOU, P. H. Salivary alterations in insulin-dependent diabetes mellitus. **Int. J. Paediatr. Dent.**, v. 8, p. 29-33, 1998.

BOUKPESSI, T.; SEPTIER, D.; BAGGA, S.; GARABEDIAN, M.; GOLDBERG, M.; CHAUSSAIN-MILLER, C. Dentin alterations of deciduous teeth in human hypophosphatemic rickets. **Calcif. Tissue Int**., v. 79, p. 294-300, 2006.

BOWE, A. E.; FINNEGAN, R.; JAN DE BEUR, S. M.; CHO, J.; LEVINE, M. A.; KUMAR, R.; SCHIAVI, S. C. FGF-23 inhibits renal tubular phosphate transport and is a PHEX substrate. **Biochem. Biophys. Res. Commun**., v. 284, p. 977-981, 2001.

BRESLER, D.; BRUDER, J.; MOHNIKE, K.; FRASER, W. D.; ROWE, P. S. Serum MEPE-ASARM-peptides are elevated in X-linked rickets (HYP): implications for phosphaturia and rickets**. J. Endocrinol**., v. 183, p. R1-9, 2004.

CHAUSSAIN-MILLER, C.; SINDING, C.; SEPTIER, D.; WOLIKOW, M.; GOLDBERG, M.; GARABEDIAN, M. Dentin structure in familial hypophosphatemic rickets: benefits of vitamin D and phosphate treatment. **Oral Dis**., v. 13, p. 482-489, 2007.

DAEP, C. A.; JAMES, D. M.; LAMONT, R. J.; DEMUTH, D. R. Structural characterization of peptide-mediated inhibition of Porphyromonas gingivalis biofilm formation. Infect. Immun., v. 74, p. 5756-5762, 2006.

ELLIOTT, J. C.; DOVER, S. D. X-ray microtomography**. J. Microsc**., v. 126, Pt. 2, p. 211-213, 1982.

FARAH, R. A.; SWAIN, M. V.; DRUMMOND, B. K.; COOK, R.; ATIEH, M. Mineral density of hypomineralised enamel. **J. Dent**., v. 38, p. 50-58, 2010.

GAUCHER, C.; WALRANT-DEBRAY, O.; NGUYEN, T. M.; ESTERLE, L.; GARABÉDIAN, M.; JEHAN, F. PHEX analysis in 118 pedigrees reveals new genetic clues in hypophosphatemic rickets. **Hum. Genet.,** v. 125, p. 401-411, 2009.

GERTNET, J. M. Metabolic bone disease. In: LIFSHITZ, F. (Ed.). **Pediatric endocrinology**. 4th ed. New York: Marcel Dekker, 2003. p. 535-536.

GIUSTI, L.; BAZZICHI, L.; BALDINI, C.; CIREGIA, F.; MASCIA, G.; GIANNACCINI, G. Specific proteins identified in whole saliva from patients with diffuse systemic sclerosis. **J. Rheumatol.,** v. 34, p. 2063-2069, 2007.

HAFFNER, D.; NISSEL, R.; WÜHL, E.; MEHLS, O. Effects of growth hormone tretament on body proportions and final height among small children with x-linked hypophosphatemic rickets. **Pediatrics**, v. 113, p. e593-e596, 2004.

HOLBROOK, I. B.; MOLAN, P. C. The identification of a peptide in human parotid saliva particularly active in enhancing the glycolitic activity of the salivary micro-organisms. **Biochem. J**., v. 149, p. 189-192, 1975.

HU, S.; YEN, Y.; ANN, D.; WONG, D. T. Implications of salivary proteomics in drug discovery and development a focus on cancer drug discovery. **Drug Discov. Today**, v. 12, p. 911-916, 2007.

IKRAM, O. H.; PATEL, S.; SAURO, S.; MANNOCCI, F. Micro-computed tomography of tooth tissue volume changes following endodontic procedures and post space preparation. **Int. Endod. J**., v. 42, p. 1071-1076, 2009.

JAGTAP, V. S.; SARATHI, V.; LILA, A. R.; BANDGAR, T.; MENON, P.; SHAH, N. S. Hypophosphatemic rickets. **Indian J. Endocrinol. Metab**., v. 16, p. 177-182, 2012.

LAWRENCE, H. P. Salivary markers of systemic disease: noninvasive diagnosis of disease and monitoring of general health. J. Can. Dent. Assoc., v. 68, p. 170-174, 2002.

LEE, K. M.; KANG, D.; YOON, K.; KIM, S. Y.; YOON, H. S.; TROUT, D. B. et al. A pilot study on the association between job stress and repeated measures of immunological biomarkers in female nurses. **Int. Arch. Occup. Environ. Health**, v. 83, p. 779-789, 2010.

LENANDER-LUMIRAKI, M.; IHALIN, R.; LÄHTEENOJA, H. Changes in whole saliva in patients with celiac disease. **Arch. Oral Biol**., v. 45, p. 347-354, 2000.

MÄKITIE, O.; DORIA, A.; KOOH, S. W.; COLE, W. G.; DANEMAN, A.; SOCHETT, E. Early tretament improves growth and biochemical and radiographic outcome in X-linked hypophosphatemic rickets. **J. Clin. Endocrinol. Metab**., v. 88, p. 3591-3597, 2003.

MALAMUD, D. Saliva as a diagnostic fluid. **Dent. Clin. North Am**., v. 55, p. 159-178, 2011.

MCWHORTER, A. G.; SEALE, N. S. Prevalence of dental abscesses in a population of children with vitamin D resistant rickets. **Pediatr. Dent**., v.13, p. 91-96, 1991.

MUGHAL, Z. Rickets in childhood. **Semin. Musculoskelet. Radiol**., v. 6, p. 183-190, 2002.

MURAYAMA, T.; IWATSUBO, R.; AKIYAMA, S.; AMANO, A.; MORISAKI, I. Familial hypophosphatemic vitamin D resistant rickets: dental findings and histology study of teeth. **Oral. Surg. Oral Med. Oral Patol. Oral Radiol. Endod**., v. 90, p. 310-316, 2000.

PEREIRA, C. M.; ANDRADE, C. R.; VARGAS, P. A.; COLETTA, R. D.; ALMEIDA, O. P.; LOPES, M. A. Dental alterations associated with x-linked hypophosphatemic rickets. **J. Endod.**, v. 30, p. 241-245, 2004.

ROOT, A. W.; DIAMOND, F. B. Disorders of calcium metabolism in the child and adolescent. In: SPERLING, M. A. (Ed.). **Pediatric endocrinology**. 2nd ed. Philadelphia: Saunders, 2002. p. 650-651.

SASHIKUMAR, R.; KANNAN, R. Salivary glucose levels and oral candidal carriage in type II diabetics. **Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod**., v. 109, p. 706-711, 2010.

SATTUR, A.; NAIKMASUR, V. G.; SHRIVASTAVA, R.; BABSHET, M. Familial hypophosphatemic rickets. **J. Indian Soc. Pedod. Prev. Dent**., v. 28, p. 302-306, 2010.

SCARANO, E.; FIORITA, A.; PICCIOTTI, P. M.; PASSALI, G. C.; CALO, L.; CABRAS, L.; INZITARI, R.; FANALI, C.; MESSANA, I.; CASTAGNOLA, M.; PALUDETTI, G. Proteomics of saliva: personal experience. **Acta Otohinolaryngol. Ital**., v. 30, p. 125-130, 2010.

SEOW, W. K. Diagnosis and management of unusual dental abscesses in children. **Aust. Dent. J.,** v. 48, p. 156-168, 2003.

SOCIEDADE BRASILEIRA DE ENDOCRINOLOGIA E METABOLOGIA. **Projeto Diretrizes:** Raquitismo Hipofosfatêmico Ligado ao X. [S.l.]: Associação Médica Brasileira/Conselho Federal de Medicina, 2004.

STRECKFUS, C. F.; BIGLER, L. R. Saliva as a diagnostic fluid. **Oral Dis**., v. 8, p. 69-76, 2002.

TOUITOU, Y.; AUZÉBY, A.; CAMUS, F.; DJERIDANE, Y. Daily profiles of salivary and urinary melatonin and steroids in healthy prepubertal boys. **J. Pediatr. Endocrinol. Metab**., v. 22, p. 1009-1015, 2009.

YONG, S. M.; AIK, S. X-Linked hypophosphatemic rickets – A report of 2 cases and review of literature. **Med. J. Malysia**, v. 55, Suppl. C, p. 101-104, 2000.

ZHANG, X.; RAHEMTULLA, F.; ZHANG, P.; LI, X.; BECK, P.; THOMAS, H. F. Normalization of calcium status reverses the phenotype in dentin, but not in enamel of VDR-deficient mice. **Arch. Oral Biol**., v. 54, p. 1105-1110, 2009.

ANEXOS

Anexo A – Aprovação do Comitê de Ética em Pesquisa com Seres Humanos

Universidade Federal do Ceará
Comitê de Ética em Pesquisa

Of. Nº 026/11 Fortaleza, 25 de fevereiro de 2011
Protocolo COMEPE nº 004/11
Pesquisador responsável: Thyciana Rodrigues Ribeiro
Título do Projeto: "Raquitismo hipofosfatêmico familiar- Estudo sobre peptídios salivares e estrutura mineral dentária"

Levamos ao conhecimento de V.Sª. que o Comitê de Ética em Pesquisa da Universidade Federal do Ceará – COMEPE, dentro das normas que regulamentam a pesquisa em seres humanos, do Conselho Nacional de Saúde – Ministério da Saúde, Resolução nº 196 de 10 de outubro de 1996 e complementares, aprovou o protocolo e o TCLE do projeto supracitado na reunião do dia 24 de fevereiro de 2011.

Outrossim, informamos, que o pesquisador deverá se comprometer a enviar o relatório final do referido projeto.

Atenciosamente,

Mirian Parente Monteiro.

Dra. Mirian Parente Monteiro
Coordenadora Adjunta do Comitê de Ética em Pesquisa
COMEPE/UFC

Anexo B: Instruções para autores da "Special Care in Dentistry"

Author Guidelines

The mission of *Special Care in Dentistry* (SCD) is to provide a forum for research findings, case reports, clinical techniques and scholarly discussion relevant to the oral health and oral health care of "special care patients." The designation of the special care patient is not limited to hospitalized, disabled or older individuals, but includes all patients for whom oral health and oral health care are complicated by physical, emotional, financial and/or access factors.

Manuscripts

Original articles are considered and accepted for publication on the condition that they have not been published or are not simultaneously submitted for publication elsewhere. A letter signed by all authors stating that the submission is an original article, not previously published or simultaneously being considered for publication elsewhere, must accompany the submission.

All manuscripts should be submitted through the online submission system athttp://mc.manuscriptcentral.com/scid.

The manuscript should be submitted with all material doublespaced, flush left (preferably in Courier typeface), with at least a 1" margin all around. All pages should be systematically numbered. The editor reserves the right to edit manuscripts to fit available space and to ensure conciseness, clarity and stylistic consistency.

Title page: Titles of articles should be descriptive but concise. Long titles discourage reading, present typographic and layout problems and create difficulties in indexing.

Include with the manuscript an abbreviated title (no more than 50 characters including punctuation and spaces) to be used on the journal cover. On the title page please include no more than 6 keywords for the article. The corresponding author should include his or her E-mail address, daytime telephone and FAX numbers, as well as current address. Positions and professional degrees should be provided along with each author's full first and last names.

Abstract: A brief structured abstract not to exceed 150 words must be included with each article and should state the following: purpose/aim of the article, the method and materials used, results and conclusions or clinical relevance.

Scientific Article Content

A critical review of the manuscript topic, the rationale and significance of the study and as appropriate study aims and/or hypotheses should be presented in the introduction.

Methods: All methods used must be detailed, referenced adequately and include a description of the statistical data analysis methods.

Results: Results must be presented in a logical order with references to appropriate tables, figures and illustrations.

Discussion: Important findings from the study should be discussed and compared with the published literature on the topic. Limitations of the study and any future research implications of the study findings must be discussed.

Conclusions: Conclusions should be presented in sentence form and not as a numerical list or dot points. Conclusions should parallel those presented in the structured abstract.

Case Report Content

Case reports should be concise and do not need to be as formally structured as scientific articles. Include a brief introduction presenting a critical literature review and a statement of the clinical implications of the case. The case description should include: personal history of the subject, socioeconomic data, health/medications history, extra-oral and intra-oral examination findings; differential diagnosis; treatment options; final treatment plan and follow-up data for a minimum of 6 months. Relevant techniques, results and data obtained should be presented. A brief discussion should reinforce the clinical implications of the case report and discuss any unique findings and insights gained, which makes this patient or patients different from any patients previously reported.

Ethics in Science

In all reports of original studies with humans, authors should specifically state the nature of the ethical review and clearance of the study protocol. Informed consent must be obtained from human subjects participating in research studies. Some reports, such as those dealing with intellectually disabled persons or institutionalized children and older adults, will need additional description of ethical clearance.

References

All references must be typed and double-spaced on a separate sheet. Authors must be listed if there are six or fewer; for seven or more authors, list the first three and add “et al.” All references given must be cited in the text and in numerical order. Bibliographies and readings lists are not used.

For journal references, give the author’s name, title of article, abbreviated journal name, volume number, inclusive pagination and year:

1. Olsen RA, Olsen DB. Hospital protocol for inpatients and outpatients. Spec Care Dentist 1987;7:257-60.

For books, give the author’s name, book title, edition(if known), location and name of publisher, inclusive pagination and year of publication:

Little JW, Falace DA. Dental management of the medically compromised patient. 2nd ed. St. Louis: Mosby; 1984:120-5.

For agency publications, give author, title, place of publication, publisher, year and publication and series numbers:

1. Jones WF III. Dental offices. Hyattsville, MD: National Center for Health Statistics, Public Health Service, National Institutes of Health;1978. DHEW publication no. (PHS)-78-1785.

For references from the web, give the source or author of the document, the title of the document, where it's available (the web site or link), and when the web site was accessed.

Special Care Dentistry Association. Who is SCDA?http://www.scdonline.org/displaycommon.cfm?an=1&subarticlenbr=73. Accessed November 27, 2007.

Tables

Tables may supplement the article with a title and should be typed on a separate sheet, numbered consecutively in Arabic numerals and cited in the text. Do not use vertical rules.

Illustrations

Illustrations include all material that cannot be set in type, such as photographs, line drawings, graphs and charts. All illustrations must be numbered and cited in the text. All illustrations must have a title and should be sent as a scanned file. Titles for graphs and charts may be placed directly above the graph or chart. Accompanying text and titles for all other illustrations should be typed and double spaced on a separate sheet, not on the illustration. Figures, charts and graphs must be drawn professionally, preferably computer-generated and laser printed.

Lettering must be large and clear. Glossy black-and-white prints of drawings must be submitted, rather than original artwork. Radiographs are not acceptable and must be submitted as glossy prints or as scanned files (eg., JPEG). All photographs and line drawings must be submitted in duplicate. Photographs should be un-mounted and untrimmed and should be high-quality, sharp, black-and-white glossy prints. On the back of each photograph, write the figure number and indicate top edge. Reproduction of color photographs is allowed and, in certain instances (particularly for some intra-oral lesions), encouraged for illustrative purposes. Additional reproductive costs for color photos will be borne by the author(s).

We are happy to receive artwork in digital format. Please save line artwork (vector graphics) as Encapsulated PostScript (EPS) and bitmap files (halftones or photographic images) as Tagged Image Format (TIFF), with a resolution of at least 300 dpi at final size. Please do not send native file formats (i.e., Excel, PowerPoint, Word, etc.). More detailed information on the submission of electronic artwork can be found at http://authorservices.wiley.com/bauthor/illustration.asp.

Permission and Waivers

These must accompany the manuscript when it is submitted for publication. Permission of author and publisher must be obtained for direct use of material (text, photographs, drawings) under copyright that is not your own (up to 100 words of prose material is typically quoted without getting permission, provided the material quoted is not the essence of the complete work). Waivers must be obtained and a copy provided for full-face photographs unless faces are masked to prevent identification.

Acknowledgements

Acknowledgements should be kept to a minimum and should specify contributors to the article other than the authors accredited.

Conflict of Interest and Source of Funding

Conflict of Interest: Authors are required to disclose any possible conflict of interest. These include financial (for example patent, ownership, stock ownership, consultancies, speaker's fee). Author's conflict of interest (or information specifying the absence of conflicts of interest) will be published under a separate heading entitled Disclosures. Any support by manufacturers or suppliers of materials and equipment must be acknowledged under the Disclosures heading.

Source of Funding: Authors are required to specify the source of funding for their research when submitting a paper. Suppliers of materials should be named and their location (town, state/county, country) included. The information will be disclosed in the published article.

Reprints

The author/s may arrange to have reprints made at their cost. Information on how to order offprints will be sent with the electronic proof from Wiley-Blackwell.

Costs

Most articles are published at no cost to the author, but special arrangements must be made with the editor for publishing articles containing extensive illustrative or tabular material or formulae or color prints.

Review Procedures

All manuscripts (except editorials, invited reviews and some commentaries) are sent by the editor to two qualified reviewers. Authors may suggest reviewers to the editor but the editor is not bound by these suggestions. If there is a conflict between the two reviewers the editor then sends the submission to a third reviewer. The reviewers' suggestions are read by the editor who, based upon the advice received, returns the manuscripts to the author/authors.

If changes are suggested by the reviewers' prior to acceptance for publication, the reviewers' comments/suggestions will be sent to the authors electronically. The authors will be asked to address all the reviewers' comments in a letter to the editor and will need to identify the page and paragraph where they have made or omitted the reviewers' comments and suggestions. If the authors choose to omit a reviewer's suggestion, they need to justify that decision in a clear and concise statement in the letter to the editor.

Submitting Accepted Article

Authors whose manuscripts have been accepted for publication will be asked to provide an electronic copy of the final draft via e-mail to SCDA@SCDAonline.org or on a disk or CD (labeled with the manuscript title, author(s), and word processing version used). There are three preferred formats for digital artwork submission: Encapsulated PostScript (EPS), Portable Document Format (PDF), and Tagged Image Format (TIFF). We suggest that line art be saved as EPS files. Alternately, these may be saved as PDF files at 600 dots per inch (dpi) or better at final size. Tone art, or photographic images, should be saved as TIFF files with a resolution of 300 dpi at final size. For combination figures, or artwork that contains both photographs and labeling, we recommend saving figures as EPS files, or as PDF files with a resolution of 600 dpi or better at final size. More detailed information on the submission of electronic artwork can be found at http://authorservices.wiley.com/bauthor/illustration.asp.

Production and Proofs

After acceptance, articles will be sent to Wiley-Blackwell to be copyedited and typeset. Then the corresponding author will receive an email with a link to the proof of his or her article. At this point, the author will need to download the proof, answer any typesetter queries, and look for any corrections that need to be made. The proofreader will mark these corrections and make her own edits. Then the typesetter will incorporate these last changes, and, after final checks are complete, the article will be published online early.

Copyrights

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

For authors signing the copyright transfer agreement

If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions http://authorservices.wiley.com/bauthor/faqs_copyright.asp

For authors choosing OnlineOpen

If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements (OAA):

Creative Commons Attribution License OAA
Creative Commons Attribution Non-Commercial License OAA
Creative Commons Attribution Non-Commercial -NoDerivs License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services http://authorservices.wiley.com/bauthor/faqs_copyright.aspand visit http://www.wileyopenaccess.com/details/content/12f25db4c87/Copyright--License.html.

If you select the OnlineOpen option and your research is funded by The Wellcome Trust and members of the

Research Councils UK (RCUK) you will be given the opportunity to publish your article under a CC-BY license supporting you in complying with Wellcome Trust and Research Councils UK requirements. For more information on this policy and the Journal's compliant self-archiving policy please visit: http://www.wiley.com/go/funderstatement.

Online Open

Online Open Online Open is available to authors of primary research articles who wish to make their article available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With OnlineOpen, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Library, as well as deposited in the funding agency's preferred archive. For the full list of terms and conditions, seehttp://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms

Any authors wishing to send their paper OnlineOpen will be required to complete the payment form available from our website at: https://onlinelibrary.wiley.com/onlineOpenOrder

Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen if you do not wish to. All OnlineOpen articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

Questions? Contact: Special Care in Dentistry, 401 N. Michigan Ave., Suite 2200, Chicago, IL 60611. Call 312.527.6764, fax 312.673.6663 or send an e-mail to SCDA@SCDAonline.org.

Anexo C: Instruções para autores do "Journal of Dental Research"

INSTRUCTIONS TO AUTHORS

The *Journal of Dental Research* (*JDR*) is a peer-reviewed scientific journal dedicated to the dissemination of new knowledge and information on all science relevant to dentistry and to the oral cavity and associated structures in health and disease. The*Journal of Dental Research's* primary readership consists of oral, dental and craniofacial researchers, clinical scientists, hard-tissue scientists, dentists, dental educators, and oral and dental policy-makers. The *Journal* is published monthly, allowing for frequent dissemination of its leading content. The *Journal of Dental Research* also offers OnlineFirst, by which forthcoming articles are published online before they are scheduled to appear in print.

Authors of all types of articles should be aware of the following guidelines when submitting to *JDR*.

ONLINE SUBMISSION

Submissions to the *Journal of Dental Research* are only accepted for consideration via the SAGETrack online manuscript submission site athttp://mc.manuscriptcentral.com/jdr. Authors who do not have an active account within the system are required to create a new account by clicking, "Create Account," on the log-in page. The system will prompt the authors through a step by step process to create their account. Once created authors can submit their manuscripts by entering their "Author Center" and clicking the button by "Click Here to Submit a New Manuscript."

If any difficulty is encountered at anytime during the account creation or submission process, authors are encouraged to contact the *Journal of Dental Research*Publications Coordinator, Kourtney Skinner, at kskinner@iadr.org.

MANUSCRIPT REQUIREMENTS BY TYPE

The *Journal of Dental Research* accepts the following types of manuscripts for consideration:
Original Research Reports: These manuscripts are based on clinical, biological, and biomaterials and bioengineering subject matter. Manuscripts submitted as research reports have a limit of 2,700 words (including abstract, introduction, materials, methods results, discussion and acknowledgments; excluding figure legends and references); a total of 4 figures or tables; 30 references; and must contain a 200 word abstract.

Letters to the Editor*: Letters must include evidence to support a position about the scientific or editorial content of the *JDR*. Manuscripts submitted as a letter to editor have a limit of 250 words. No figures or tables are permitted. Letters on published articles must be submitted within 3 months of the article's print publication date.

Guest Editorials*: A clear and substantiated position on issues of interest to the readership community can be considered for this manuscript type. Guest Editorials are limited to 1,000 words. No figures or tables are permitted.

Discovery!: Essays that explore seminal events and creative advances in the development of dental research are considered for the "Discovery!" section of the*Journal*. Manuscripts submitted for "Discovery!" have a limit of 2,500 words and a total of 2 figures or tables. Manuscripts are to be submitted by invitation only. Questions regarding "Discovery!" should be directed to Dr. Marty Taubman, atmtaubman@forsyth.org.

***Critical Reviews in Oral Biology & Medicine*:** These manuscripts should summarize information that is well known and emphasize recent developments over the last three years with a prominent focus on critical issues and concepts that add a sense of excitement to the topic being discussed. Manuscripts are to be submitted by invitation only. Authors interested in submitting to this section must contact the Editor of *Critical Reviews in Oral Biology & Medicine*, Dr. Dana Graves, atgravesdt@umdnj.edu for submission approval and instructions. Manuscripts submitted as Critical Reviews have a limit of 4,000 words; a total of 6 figures or tables; 60 references; and must contain a 200 word abstract.

Additional Instructions for Critical Reviews:
-It is important to include several illustrations or diagrams to enhance clarity. Manuscripts that lack figures or diagrams typically receive a low priority score.

-Summarize important concepts in tables or flow charts or show critical data in the form of figures. NOTE: authors will need to obtain permission to reproduce a previously published figure or table.

-Due to the broad readership, abbreviations commonly recognized in one field may not be readily apparent to those in a different field. Keep abbreviation use to a minimum.

-The cover page, abstract, text, summary, figure legends, and tables should be combined into a single Word document. Figures should be submitted as a separate document.

-To view examples of recent Critical Reviews in the *Journal*, please click the following links: http://jdr.iadrjournals.org/cgi/content/full/86/9/800 orhttp://jdr.iadrjournals.org/cgi/content/full/85/7/584

***Brief responses to Letters to the Editor or Guest Editorials will be solicited for concurrent publication.**

Clinical Reviews (formerly Concise Reviews): These manuscripts are generally systematic reviews of topics of high clinical relevance to oral, dental and craniofacial research. Meta-analyses should be considered only when sufficient numbers of studies are available. Manuscripts that include investigations of limited study quality of under-studied areas are typically not acceptable as topics for a clinical review. Although some systematic reviews may be well done, those that receive highest scientific priority will only be considered given the very limited space allowed for these reviews in the *Journal*. Pre-submission inquiries for clinical reviews must contact the Editor-in-Chief, Prof. William Giannobile, william.giannobile@umich.edu for submission approval and instructions. Manuscripts submitted as Clinical Reviews have a strict limit of 4,000 words (including abstract, and the main text of the manuscript including acknowledgments; excluding figure legends and references); a total of 6 figures or tables; up to a maximum of 60 references; and must contain a 200 word abstract. Manuscripts above the 4,000 word/6 figure or table limit may use supplemental appendices for other supporting information that would be available online only.

Additional Instructions for Clinical Reviews:
-It is important to include illustrations or diagrams to enhance clarity. Manuscripts that lack figures or diagrams typically receive a low priority score.

-Summarize important concepts in tables or flow charts or show critical data in the form of figures. NOTE: authors will need to obtain permission to reproduce a previously published figure or table.

-Due to the broad readership, abbreviations commonly recognized in one field may not be readily apparent to those in a different field. Keep abbreviation use to a minimum.

-The cover page, abstract, text, summary, figure legends, and table(s) should be combined into a single Word document. Figures should be submitted as a separate document.

-To view examples of recent Clinical Reviews in the *Journal*, please click the following links: http://jdr.sagepub.com/content/90/3/304.full.pdf+html orhttp://jdr.sagepub.com/content/90/5/573.full.pdf+html

All submissions must include a title page and be accompanied by a cover letter and list of suggested reviewers. Cover letters should certify the research is original, not under publication consideration elsewhere, and free of conflict of interest. Title pages should include: abstract word count, total word count (Abstract to Acknowledgements), total number of tables/figures, number of references, and a minimum of 6 keywords. Keywords cannot be words that have been included in the manuscript title. Key words should be included selected from Medical Subject Headings (MeSH) to be used for indexing of articles. See:http://www.nlm.nih.gov/mesh/MBrowser.html for information on the selection of key words.

Please submit the names and email addresses of four preferred reviewers when prompted by the SAGETrack system. Preferred reviewers cannot be colleagues at the contributors' institution or present or former collaborators.

TITLES

Titles can consist of a maximum of 75 characters (including spaces). Titles do not normally include numbers, acronyms, abbreviations or punctuation. The title should include sufficient detail for indexing purposes but be general enough for readers outside the field to appreciate what the paper is about.

ACKNOWLEDGEMENTS

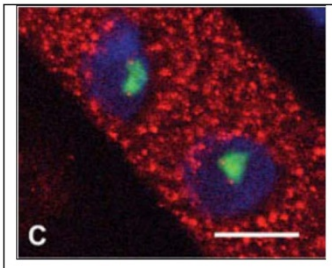
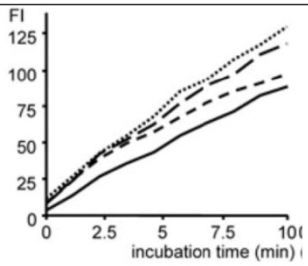
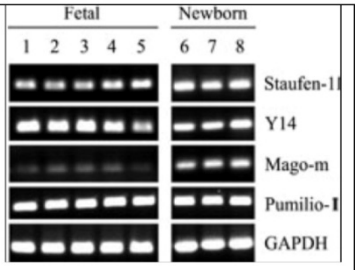
Authors are required to report all sources of support for their project or study, including but not limited to: grant funds, commercial sources, funds from a contributors' institution. Do not refer to a study being "partially funded by the cited sources." Consultancies and funds paid directly to investigators must also be listed. Authors are required to specify during the submission process if their paper received funding from NIH, NIDCR, or any other NIH Institute or Center and provide the grant number. To comply with the NIH Public Access Mandate, for qualifying NIH-funded papers, the *Journal of Dental Research* will deposit the final, copyedited paper to PubMed Central on behalf of the authors.
Any perceived or actual conflicts of interest need to be identified in the acknowledgments section. The *JDR* abides by the International Committee of Medical Journal Editors guidelines for the Ethical Considerations in the Conduct and Report of Research (http://www.icmje.org/ethical_4conflicts.html). Authors are requested to include this information in the acknowledgments section and the corresponding author must confirm that all co-authors have reported any potential conflicts.

FIGURE AND TABLE REQUIREMENTS

These guidelines are intended to aid authors in providing figures that will reproduce well in both print and online media. Submitting digital image files that conform to these guidelines will prevent delays in the review and publication processes, and maximize the published quality of your figures.

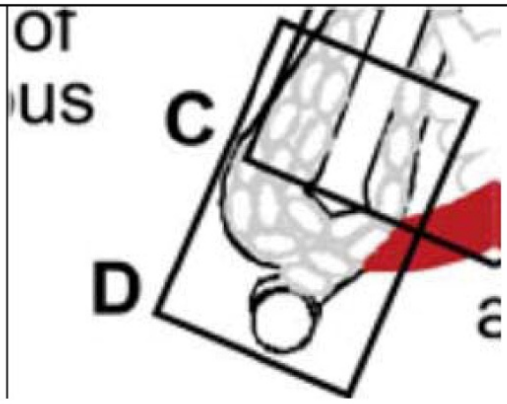
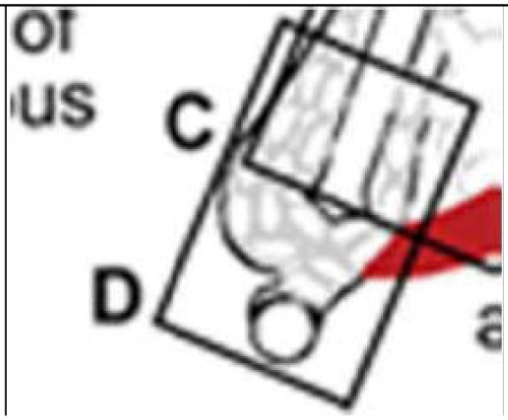
Figure Types

JDR figures can fall into one of three categories: **Continuous-tone images, Line-art images**, and **Combination images**. Each image type has specific requirements in terms of the resolution needed for publication and the file types best suited for the figure. See the following panels for examples and requirements.

		
Continuous-tone Image Minimum resolution: 300dpi. Preferred File Formats: TIFF, Bitmap.	**Line-art Image** Minimum resolution: 800dpi. Preferred File Formats: EPS, PowerPoint, Illustrator.	**Combination Image** Minimum resolution: 800dpi. Preferred File Formats: PDF, EPS, PowerPoint, Illustrator, InDesign.

Resolution

In order for a figure to be used in publication, its Digital Image File must have the required resolution when it is created. The resolution cannot be raised after the original image is made. Attempting to do so (for example, with Adobe Photoshop's© "*Image Size*" command) results in the addition of artificial pixels that distort the image and lower its sharpness. The figures on the right show an example of this reduced sharpness.

	
Line-art supplied at high resolution (1000dpi).	Using "Image Size" to go from 300 DPI to 1000 DPI.

Fonts

Limit fonts used in any figure to Times, Times New Roman, Arial, Frutiger, and Sabon. Other fonts cannot be guaranteed to reproduce properly.

Files containing figures and tables should be clearly labeled to indicate their placement in the text or appendix. Tables should be viewable in a portrait view. Tables that are created in a landscape view are more suitable for an appendix.

If the online version is in color and the printed version in black and white, please submit separate files for each version. Figures should be identical except in color or grayscale. The cost of color figures in the print version will be borne by the authors. Rates for color reproduction are $300 per initial page of color and $150 for each additional page of color. However, there are no charges for figures and diagrams printed in black and white. Color figures many be included in the online version of *JDR*with no extra charges.

REFERENCES

Citations should be arranged in alphabetical order by last name of the first author without numbering. When citing a reference in the text, provide attribution for the subject under discussion. “Et al” should be used when the cited work is by six or more contributors. When the cited work is by two contributors, use both surnames cited in the following manner: Last Name1, First Name1, Last Name2, First Name2. When citing multiple references by the same author(s) in the same year, use “a,” “b,” etc. (e.g., Jones, 1980b). Multiple references should be listed in chronological order of publication, separated by semi-colons. Avoid using abstracts as references.

When citing a Website, list the authors and title if known, then the URL, include the date it was accessed in parentheses. Include among the reference papers accepted but not yet published; designate the journal and add “in press.” Information from manuscripts submitted but not yet accepted should be cited in the text as “unpublished observations” in parentheses. The references must be verified by the author(s) against the original documents and checked for correspondence between references cited in the text and listed in the “References” section.

All items should be listed alphabetically by the author’s last name. For multiple entries by the same author/authors, they should be cited as follows:
1. One author: chronologically by year of publication
2. Two authors: alphabetical by last names
3. Three or more authors: chronologically by year of publication
4. Same author, same publication, chronologically by date of publication, using a), b), etc., to designate order

“Unpublished observations” and “personal communications” may be inserted into and cited in the text with written permission from the correspondents, but are not to be used as references.

For examples of reference citation formats, please click here.

SUPPLEMENTAL FILES

Additional supporting data may be referenced as a supplemental appendix for publication online only. All supplemental appendix files must be submitted with the manuscript for review. Supplemental files may include additional figures or tables that exceed the *Journal’s* limit. Material intended for the supplemental appendix must have “supplemental” or “appendix” in the file name upon upload.
For additional information on formatting manuscripts please click here to be directed to a detailed description of required manuscript components.
Language Editing: Manuscripts submitted for publication consideration should be written in English. Prior to submission, if a manuscript would benefit from professional editing, authors may consider using a language-editing service. Suggestions for this type of service can be found at www.iadr.org/EditingServices. The *Journal of Dental Research* does not take responsibility for, or endorse these services, and their use has no bearing on acceptance of a manuscript for publication.

GENERAL INFORMATION FOR AUTHORS SUBMITTING A MANUSCRIPT

PRIOR PUBLICATION

Manuscripts submitted to the *Journal of Dental Research* are accepted for consideration giving the understanding that it contains original material that has not been submitted for publication or has been previously published elsewhere. Any form of publication other than an abstract only constitutes prior publication.

ICJME COMPLIANCE STATEMENT

Manuscript submission guidelines for the *Journal of Dental Research* follow the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" set forth by the International Committee of Medical Journal Editors (ICJME). For additional information please visit the ICMJE website at http://www.icmje.org/.

CONSORT 2010 CHECKLIST COMPLETION RANDOMIZED CLINICAL TRIALS POLICY

Manuscripts reporting a randomized clinical trial should follow the CONSORT guidelines.

The *Journal* requires authors of pre-clinical animal studies submit with their manuscript the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines. Authors of human observations studies in epidemiology are required to review and submit a STROBE statement. When uploaded to the SAGETrack system, any checklists completed by authors should be given a supplementary file designation.

The CONSORT checklist can be downloaded from:http://mc.manuscriptcentral.com/societyimages/jdr/CONSORT+2010+checklist%5b1%5d.doc.

The ARRIVE guidelines can be found here: www.nc3rs.org.uk/downloaddoc.asp?id=1206&page=1357&skin=0

The STROBE checklists can be found here: www.strobe-statement.org/index.php?id=strobe-home

The *Journal of Dental Research* encourages authors to register their clinical trials in a public trials registry. Authors of manuscripts describing such studies are asked to submit the name of the registry and the study registration number prior to publication. In accordance with the aforementioned "Uniform Requirements for Manuscripts Submitted to Biomedical Journals," clinical trials will only be considered for publication if they are registered.

INSTITUITIONAL REVIEW BOARD AND WRITTEN INFORMED CONSENT

For protocols involving the use of human subjects, authors should indicate in their Methods section that subjects' rights have been protected by an appropriate Institutional Review Board and written informed consent was granted from all subjects. When laboratory animals are used, indicate the level of institutional review and assurance that the protocol ensured humane practices.

PUBLIC GENE DATA

Prior to submission, the *Journal of Dental Research* asks that novel gene sequences be deposited in a public database and the accession number provided to the *Journal*. Authors may want to use the following *Journal* approved databases:

GenBank: http://www.ncbi.nlm.nih.gov/Genbank/submit.html

EMBL: http://www.ebi.ac.uk/embl/Submission/index.html

DDBJ: http://www.ddbj.nig.ac.jp/sub-e/html

Manuscript submissions including microarray data should include the information recommended by the MIAME guidelines in their submission, and/or identify the submission details for the experiments details to one of the publicly available databases such as ArrayExpress or GEO.

OPEN ACCESS COMPLIANCE STATEMENT

Effective April 7, 2008 the National Institutes of Health (NIH) Revised Policy on Enhancing Public Access to Archived Publications Resulting from NIH-Funded Research (Public Access Policy) requires all studies funded by NIH to submit or have submitted for them their final peer-reviewed manuscript upon acceptance for publication to the National Library of Medicine's PubMed Central (PMC) to be made publicly available no later than 12 months after the official date of publication. The*Journal of Dental Research* adheres to the Washington DC Principles for Free Access to Science and makes all content available after 12 months. Only final, copyedited manuscripts are uploaded.

Manuscripts by authors whose work is funded by the Wellcome Trust will be released from access control immediately upon publication. Members of the IADR/AADR will be invoiced $3,800; non-Members will be billed $4,300 for this immediate access. No separate page or color charges will be assessed.

Authors are required to specify during the submission process if their paper received funding from NIH or NIDCR and provide the grant number.

The *Journal of Dental Research* will deposit final, copyedited papers to PubMed Central on behalf of the authors.

DEFINITION OF CONTRIBUTORSHIP IN *JDR*

As stated in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, put forth by the ICJME, the *Journal* considers the following as an accurate definition of contributorship:

Contributors Listed in Acknowledgments

As stated in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, put forth by the ICJME, the *Journal* considers the following as an accurate definition of contributorship:

Contributors Listed in Acknowledgments

All contributors who do not meet the criteria for authorship should be listed in an acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chairperson who provided only general support. Editors should ask corresponding authors to declare whether they had assistance with study design, data collection, data analysis, or manuscript preparation. If such assistance was available, the authors should disclose the identity of the individuals who provided this assistance and the entity that supported it in the published article. Financial and material support should also be acknowledged.

Groups of persons who have contributed materially to the paper but whose contributions do not justify authorship may be listed under such headings as “clinical investigators” or “participating investigators,” and their function or contribution should be described—for example, “served as scientific advisors,” “critically reviewed the study proposal,” “collected data,” or “provided and cared for study patients.” Because readers may infer their endorsement of the data and conclusions, these persons must give written permission to be acknowledged.

CONTRIBUTOR FORMS

All rights to manuscripts will be transferred to the *Journal of Dental Research* upon submission. Submission of a manuscript will constitute each author’s agreement that the *Journal* holds all propriety rights in the manuscript submitted, including all copyrights. Upon acceptance, contributors will be asked to sign a formal transfer of copyright.

Please note that the *Journal of Dental Research* secures completed contributor forms electronically via the SAGETrack online submission and review system. It is important to provide a valid email address for all co-authors listed at the time of submission. In the instance of manuscript acceptance for publication in the *Journal*, these email addresses will be used to distribute the contributor forms to co-authors.

Without the completion of the contributor forms for all co-authors listed, accepted manuscripts cannot continue into production, delaying publication.

CHARGES ASSOCIATED WITH PUBLICATION

Page Charges

There is a charge of $40 (U.S.) for every printed page in the *Journal of Dental Research*. There is a charge of $25 (U.S.) for every electronic page in a Web appendix. You will receive an invoice with your page proofs.

Color Figure Charges

The cost of color figures in the print version will be borne by the authors. Rates for color reproduction are $300 per initial page of color and $150 for each additional page of color. However, there are no charges for figures and diagrams printed in black and white. Color figures many be included in the online version of *JDR* with no extra charges.

Reprint Charges

Reprints can be ordered for material printed in the *Journal of Dental Research* and online only appendices. Quantities of reprints can be purchased with the reprint order form sent with page proofs to the contributors. Pre-payment is required for reprints. Visa, MasterCard, American Express and check are all acceptable forms of payment. Authors must pay for color figures in reprints. Reprints will be mailed from 6 to 8 weeks after the article appears in the *Journal*. To contact SAGE for additional information or to order reprints, click here.

Anexo D: Instruções para autores do "The Journal of Clinical Endocrinolog and Metabolism"

Instructions to Authors for *The Journal of Clinical Endocrinology & Metabolism*

Purpose and Scope

The Journal of Clinical Endocrinology & Metabolism (JCEM) publishes original research articles, reviews, and other special features related to endocrinology and metabolism in humans and human tissue.

back to top

Expectation of Ethical Conduct

The Endocrine Society's mission is to advance excellence in endocrinology and be an integrative force in scientific research and medical practice. Such progress depends on integrity in the conduct of scientific research and truthful representation of findings. Specific guidelines regarding the Society's expectations for ethical conduct can be found in the Code of Ethics of The Endocrine Society and the Ethical Guidelines for Publications of Research.

The journal editors and publication oversight committees of The Endocrine Society are dedicated to upholding high ethical standards in its publications and expect authors and reviewers to do the same.

back to top

General Information

Manuscripts must be written in idiomatic English and conform to the specifications described below. Papers that do not meet these requirements will be returned to the author for necessary revision before formal review. Manuscripts submitted to *JCEM* are usually evaluated by peer reviewers who remain anonymous; but the disposition of some manuscripts is determined by the editors alone. Authors of manuscripts requiring modifications have two (2) months to resubmit a revision of their paper. Manuscripts returned after more than two (2) months will be treated as new submissions. An unsolicited revision of a rejected manuscript will either be returned or treated as a new submission, at the editor's discretion.
In response to a growing demand for online content, the JCEM is posting three types of articles online only: Brief Reports, Hot Topics in Translational Endocrinology, and Advances in Genetics. The last two categories are chosen by the editors upon acceptance (see Dr. Wartofsky's Editorial) .
All papers accepted during each publishing year are eligible for The Endocrine Society and Pfizer, Inc. International Award for Excellence in Published Clinical Research in The Journal of Clinical Endocrinology & Metabolism (information at http://www.endo-society.org/awards/JournalAwards/index.cfm).

back to top

Manuscript Categories

Reports of original research may be submitted to *JCEM* as an Original Article or Brief Report. Other special categories of manuscripts are described below. All manuscripts must adhere to the word count limitations, as specified below, for text only; the word count does not include the abstract, references, or figure/table legends. The word count must be noted on the title page, along with the number of figures and tables.

- **Original Articles** should be no longer than 3600 words and include no more than six figures and tables and 40 references. The Journal has a special interest in publishing results of major prospective randomized clinical trials, which may be eligible for submission through *Endocrine Trials Express*, a pathway for expedited manuscript review that aims to provide an initial editorial decision within two weeks. Authors who wish to request consideration by *Endocrine Trials Express* should contact the Managing Editor by e-mail (sherman@endo-society.org) before submitting their paper.
- **Brief Reports** are succinct descriptions of focused studies with important, but very straightforward, negative or confirmatory results. These manuscripts should be no longer than 1800 words and include no more than two figures and tables and 20 references.
- **Clinical Reviews** and other **Reviews** should address topics of importance to clinical endocrinologists and endocrine clinical investigators, including scholarly updates regarding the molecular and biochemical basis for normal physiology and disease states; the state-of-the-art in diagnosis and management of endocrine and metabolic disorders; and other topics relevant to the practice of clinical endocrinology. Authors considering the submission of uninvited reviews should contact the editors in advance to determine whether the topic that they propose is of current potential interest to the Journal. These manuscripts should be no longer than 4000 words and include no more than four figures and tables and 120 references. Authors should include a brief section describing the search strategies used to obtain information for the review.

- **Clinical Case Seminars** are descriptions of a case or small number of cases revealing novel and important insights into a condition's pathogenesis, presentation, and/or management. The case report is to be accompanied by a concise scholarly review of the literature regarding relevant aspects of the disorder. These manuscripts should be 2400 words or less, with no more than four figures and tables and 30 references.
- **Extensive Clinical Experiences** are learned descriptions of substantial clinical experience with a specific endocrine or metabolic disorder, or class of disorders, by a single clinical endocrinologist or facility. This experience should expose novel aspects of the condition's presentation, diagnosis, natural history, and/or treatment. These manuscripts should be no longer than 3600 words and include no more than four figures and tables and 40 references.
- **Position** and **Consensus Statements** related to the endocrine and metabolic health standards and healthcare practices may be submitted by professional societies, task forces, and other consortia. All such submissions will be subjected to peer review, must be modifiable in response to criticisms, and will be published only if they meet the Journal's usual editorial standards. These manuscripts should typically be no longer than 3600 words and include no more than six figures and tables and 120 references.
- **Controversies in Clinical Endocrinology** describe and justify different approaches to diagnosis and/or management of patients with an endocrine or metabolic condition. This feature typically consists of a pair of manuscripts authored by two individuals who thoughtfully describe their respective clinical perspectives on a problem, their related practices, and the rationale and evidence supporting them. The entire manuscript should be no longer than 2400 words and include no more than two figures and tables and 30 references.
- **Images in Endocrinology** are to be comprised of a single figure or two closely related figures that illustrate the value of visual information in clinical diagnosis of endocrine and metabolic disorders, with a caption that is 50 words or less, an accompanying commentary that is 250 words or less, and five or fewer references.
- **Commentaries** are essentially uninvited editorials, which should concisely address and take a well-reasoned position on a timely issue of importance to clinical endocrinologists and/or endocrine clinical investigators. These manuscripts should be no longer than 1200 words with no more than 10 references; no figures or tables are permitted.
- **Letters to the Editor** may be submitted in response to work that has been published in the Journal. **Letters**should be short commentaries related to specific points of agreement or disagreement with the published work.**Letters** are not intended for presentation of original data unrelated to a published article. **Letters** can only be submitted electronically via the Journal website, by clicking on the link entitled "Submit a Letter to the Editor" on the abstract page or the article itself. Letters should be no longer than 500 words with no more than five complete references, and may not include any figures or tables.

back to top

Manuscript Submission Procedures

JCEM only uses electronic manuscript submission at Editorial Manager (http://jcem.edmgr.com).

If this is your first submission to an Endocrine Society Journal, click on "Register Now" to create an author account. If you already have an account from a previous submission to any of The Endocrine Society's Journals, enter your username and password to submit a new or revised manuscript. If you have forgotten your username and/or password, e-mail the editorial office (sherman@endo-society.org) for assistance.

Note that your author account is the same for *JCEM*, *Endocrinology*, *Molecular Endocrinology*, and *Endocrine Reviews*. Authors should be aware that in submitting a manuscript for consideration by *JCEM*, they are submitting their paper to The Endocrine Society Central Journals Office database, which is accessible by the Editors-in-Chief of all the Society's journals.

All submissions must include:

- A cover letter requesting that the manuscript be evaluated for publication in *JCEM* and any information relevant to your manuscript. Elsewhere on the submission form authors may suggest up to five specific reviewers and/or request the exclusion of up to three others.
- Assignment of Copyright and Disclosure of Potential Conflict of Interest is part of the online submission process. At the time of submission all co-authors will receive authorship verification emails to which they must respond. It is imperative that all co-authors are listed on the submission forms and their email address be correct.
- At least three key terms.
- Completed Disclosure Summary on the title page. For instructions on preparing the summary, see the following page (http://jcem.endojournals.org/site/author/RequiredForms.pdf).
- Authors are encouraged to submit a PDF for the initial submission. See the instructions on the JCEM homepage. If you do submit original files, Editorial Manager will create a PDF of your files, but it may take some time depending on the size of the files.

back to top

Manuscript Preparation

General Format

The Journal requires that all manuscripts be submitted in a single-column format that follows these guidelines:

- All text should be double-spaced with 1-inch margins on both sides using 11-point type in Times Roman font.
- All lines should be numbered throughout the entire manuscript and the entire document should be paginated.
- All tables and figures must be placed after the text and must be labeled. Submitted papers must be complete, including the title page, abstract, figures, and tables. Papers submitted without all of these components will be placed on hold until the manuscript is complete.
- Authors are encouraged to cite primary literature rather than review articles in order to give credit to those who have done the original work.
- Any supplemental data intended for publication must meet the same criteria for originality as the data presented in the manuscript.

back to top

Title Page

The title page should include the following:

- Full title (a concise statement of the article's major contents)
- Authors' names and institutions. At least one person must be listed as an author; no group authorship without a responsible party is allowed. A group can be listed in the authorship line, but only on behalf of a person or persons. All group members not listed in the authorship line must be listed in the Acknowledgments.
- Abbreviated title of not more than 40 characters for page headings
- At least three key terms for indexing and information retrieval
- Word count (excluding abstract, figure captions, and references)
- Corresponding author's e-mail and ground mail addresses, telephone and fax numbers
- Name and address of person to whom reprint requests should be addressed
- Any grants or fellowships supporting the writing of the paper
- Disclosure summary (see Disclosure of Potential Conflict of Interest form for instructions)
- Clinical Trial Registration Number, if applicable

back to top

Structured Abstracts

All Original Articles, Brief Reports, Clinical Reviews, Clinical Case Seminars, Consensus and Position Statements, Controversies in Endocrinology, and Extensive Clinical Experiences should be submitted with structured abstracts of no more than 250 words. All information reported in the abstract must appear in the manuscript. The abstract should not include references. Write the abstract with a general medical audience in mind. Please use complete sentences for all sections of the abstract.

Detailed instructions on writing Structured Abstracts are at http://jcem.endojournals.org/site/misc/Structured_Abstracts.xhtml.

back to top

Introduction

The article should begin with a brief introductory statement that places the work to follow in historical perspective and explains its intent and significance.

back to top

Materials and Methods

These should be described and referenced in sufficient detail for other investigators to repeat the work. The source of hormones, unusual chemicals and reagents, and special pieces of apparatus should be stated. For modified methods, only the modifications need be described.

back to top

Results and Discussion

The Results section should briefly present the experimental data in text, tables, and/or figures. For details on preparation of tables and figures, see below. The Discussion should focus on the interpretation and significance of the findings with concise objective comments that describe their relation to other work in that area. The Discussion should not reiterate the Results.

back to top

Acknowledgments

The Acknowledgments section should include the names of those people who contributed to a study but did not meet the requirements for authorship. The corresponding author is responsible for informing each person listed in the acknowledgment section that they have been included and providing them with a description of their contribution so they know the activity for which they are considered responsible. Each person listed in the acknowledgments must give permission - in writing, if possible - for the use of his or her name. It is the responsibility of the corresponding author to collect this information.

back to top

References

References to the literature should be cited in numerical order (in parentheses) in the text and listed in the same numerical order at the end of the manuscript on a separate page or pages. The author is responsible for the accuracy of references. The number of references cited should be limited, as indicated above for each category of submission. Appropriate recent reviews should be cited whenever possible.

Examples of the reference style that should be used are given below. Further examples will be found in the articles describing the Uniform Requirements for Manuscripts Submitted to Biomedical Journals (Ann Intern Med.1988; 108:258-265, Br Med J. 1988; 296:401-405). The titles of journals should be abbreviated according to the style used in the *Index Medicus*.

Journal articles and abstracts: List all authors. The citation of unpublished observations, of personal communications, and of manuscripts in preparation or submitted for publication is not permitted in the bibliography. Such citations should be inserted at appropriate places in the text, in parentheses and without serial number, or be presented in the footnotes. The citation of manuscripts accepted for publication but not yet in print is permitted in the bibliography provided the DOI (Digital Object Identifier) and the name of the journal in which they appear are supplied. Listing a manuscript as "in press" without a DOI and journal title is not permitted. If references to personal communications are made, authors are encouraged to keep written proof of the exchange. If it is necessary to cite an abstract because it contains substantive data not published elsewhere, it must be designated at the end of the reference [e.g., 68:313 (Abstract)].

Books: List all authors or editors.

Sample References

1. **Binoux M, Hossenlopp P** 1986 Insulin-like growth factor (IGF) and IGF-binding proteins: comparison of human serum and lymph. J Clin Endocrinol Metab 67:509-514
2. **MacLaughlin DT, Cigarros F, Donahoe PK** 1988 Mechanism of action of Mullerian inhibiting substance. Program of the 70th Annual Meeting of The Endocrine Society, New Orleans, LA, 1988, p 19
3. **Bonneville F, Cattin F, Dietemann J-L** 1986 Computed tomography of the pituitary gland. Heidelberg: Springer-Verlag; 15-16
4. **Burrow GN** 1987 The thyroid: nodules and neoplasia. In: Felig P, Baxter JD, Broadus AE, Frohman LA, eds. Endo crinology and metabolism. 2nd ed. New York: McGraw-Hill; 473-507

For general aid in the preparation of manuscripts, authors should consult: CBE Style Manual: A Guide for Authors, Editors and Publishers. 5th ed. Bethesda, MD: Council of Biology Editors; 1983.

back to top

Tables

Tables must be constructed as simply as possible and be intelligible without reference to the text. Each table must have a concise heading. A description of experimental conditions may appear together with footnotes at the foot of the table. Tables must not simply duplicate the text or figures. The width of the table must be designed to occupy one or two journal columns, with no more than four table columns or 8-10 table columns, respectively.

back to top

Figures and Legends

Please review the detailed instructions for preparing digital art at http://art.cadmus.com/da/index.jsp. E-mail queries can be sent todigitalart@cadmus.com. All figures must display the figure number.

Sizing the figure: The author is responsible for providing digital art that has been properly sized, cropped, and has adequate space between images. Plan the size of the figure to fill 1, 1.5, or 2 columns in the printed journal (see chart below for dimensions). In most cases, figures should be prepared for 1-column width. Produce original art at the size it should appear in the printed journal. (Note for PowerPoint users: The sizing instructions do not apply if you are submitting PowerPoint files for print production in Editorial Manager. On the submission page, check boxes to indicate that the figures are the correct size and resolution.)
1 column = 18 picas, 7.5 cm, 3.0 in
1.5. columns = 30 picas, 12.5 cm, 5.0 in
2 columns = 38 picas, 16.0 cm, 6.5 in

Lettering: At 100% size, no lettering should be smaller than 8 point (0.3 cm high) or larger than 12 point (0.4 cm high). Use bold and solid lettering. Lines should be thick, solid, and no less than 1-point rule. Avoid the use of reverse type (white lettering on a darker background). Avoid lettering on top of shaded or textured areas. Titles should be clear and informative. Keep wording on figures to a minimum, and confine any explanation of figures to their separate-page legends. Label only one vertical and one horizontal side of a figure. *Freehand lettering or drawing is unacceptable*.

Color Figures: Figures should now be submitted as RGB (red, green, blue) format. Saving color figures to this format will be more convenient for authors as RGB is the standard default on most programs. Color images will be preserved as RGB up until the time of printing and will be posted online in their original RGB form. Using RGB color mode for online images will be a significant improvement for figures that contain fluorescent blues, reds, and greens. Therefore the online journal will accurately reflect the true color of the images the way the author intended. For print, the images will be converted to CMYK through an automated color conversion process.

Shading: Avoid the use of shading, but if unavoidable, use a coarse rather than a fine screen setting

(80-100 line screen is preferred). Avoid 1-20% and 70-99% shading; make differing shades vary by at least 20%, *i.e.*, 25%, 45%, 65%. Instead of shading, denote variations in graphs or drawings by cross-hatching; solid black; or vertical, horizontal, or diagonal striping. Avoid the use of dots.

Grouped figures: For grouped figures, indicate the layout in a diagram. Place grouped figures so that they can be printed in 1 column width with uniform margins. Indicate magnification in the legends and by internal reference markers in the photographs. Their length should represent the fraction or multiple of a micrometer, appropriate to the magnification.

Graphs: Graphs with axis measures containing very large or small numbers should convert to easily readable notations. *Example*: For an ordinate range of "counts per minute" values from 1,000 to 20,000, the true value may be multiplied by 10^{-3} (scale would read from 1 to 20) and the ordinate axis display "cpm ($x10^{-3}$)." Similarly, for a Scatchard plot with values ranging from 0.1 to 2 femtomolar (10^{-15} m), the scale may run from 0.1 to 2 with the abscissa labeled "m ($x10^{-15}$)." *Three-dimensional bar graphs will not be published if the information they refer to is only two-dimensional.*

back to top

Supplemental Data

Supplemental Data allows authors to enhance papers in *JCEM* by making additional substantive material available to readers. Supplemental Data may take the form of figures, tables, datasets, derivations, or videos, and is published only in *JCEM* online; it does not appear in the printed version of the journal. Authors who wish to include Supplemental Data should state so in the cover letter when the manuscript is submitted.

Supplemental Data files should be submitted through Editorial Manager at the time of manuscript submission, and will be reviewed along with the manuscript. The files should be uploaded in the field marked "Upload Supplemental Data Files", and should NOT be attached with the manuscript and figure files. Authors should refer to the Supplemental Data in the manuscript at an appropriate point in the text or figure/table legend.

The file formats listed below may be used for Supplemental Data. Provide a brief description of each item in a separate HTML or Word file (*i.e.*, figure or table legends, captions for movie or sound clips, etc.). Do not save figure numbers, legends, or author names as part of an image. File sizes should not exceed 5 MB. Images should not exceed 500 pixels in width or height. Do not use tabs or spaces for Word or WordPefect tables; please use the table functions available within these word processing programs to prepare tables. For web pages, provide a complete list of files and instructions for creating directories.

.htm, HTML*
.jpg, JPEG image*
.gif, Graphical image
.pdf, Adobe Portable Document Format
.xls, MS Excel Spreadsheet
.mov, Quick Time
.wav, Sound
.doc, MS Word 6 documents**
.txt, Plain ASCII*

*These files can be viewed directly on standard web browsers.
**MS Word may be used for text only.

back to top

Units of Measure

Results should be expressed in metric units. Systeme Internationale (SI units) must be added in parentheses. Temperature should be expressed in degrees Celsius (*e.g.*, 28 C) and time of day using the 24-hour clock (*e.g.*, 0800 h, 1500 h).

back to top

Standard Abbreviations

All nonstandard abbreviations in the text must be defined immediately after the first use of the abbreviation. The list of **Standard Abbreviations** is given in the link.

back to top

Editorial Policies and Guidelines

Prior Publication

Failure to notify the editor that some results in the manuscript are being or have been previously published will result in placement of a notice in the journal that the authors have violated the Ethical Guidelines for Publication of Research in The Endocrine Society Journals. The journal publishes original research and review material. Material previously published in whole or in part shall not be considered for publication. This includes materials published in any form of mass communication. At the time of submission, authors must divulge in their cover letter all prior publications or postings of the material in any form of media. Abstracts or posters displayed for colleagues at scientific meetings need not be reported. Other postings of any part of the submitted material on web pages, as well as those essential for participation in required registries will be evaluated by the Editor-In-Chief, who shall determine if those postings are material enough to constitute prior publication.

back to top

Authorship Criteria

An author should have participated in either the conception, planning, or execution of the work, the interpretation of the results and the writing of the paper. An acknowledgment accompanying the paper is appropriate recognition for others who have contributed to a lesser extent, e.g., provision of clones, antisera or cell lines, or reading and reviewing manuscripts in draft. The signature of each author on the Affirmation of Originality and Copyright Release form that must be submitted with the manuscript indicates that all authors have had a part in the writing and final editing of the report, all have been given a copy of the manuscript, all have approved the final version of the manuscript, and all are prepared to take public responsibility for the work, sharing responsibility and accountability for the results. Medical writers can be legitimate contributors, and their roles, affiliations, and potential conflicts of interest should be described when submitting manuscripts. These writers should be acknowledged on the byline or in the Acknowledgments section in accord with the degree to which they contributed to the work reported in the manuscript. Failure to acknowledge these contributors would mean that the manuscript could have been "ghost-written," which is not allowed.

back to top

Guidelines for considering authors of non-research articles who have a potential COI

The editors of The Endocrine Society's journals appreciate the importance of assuring unbiased authorship of editorials, reviews, and other non-research features involving selection of evidence to be discussed and perspectives to be presented. Consequently, special care is taken in choosing authors for such articles to assure their views are balanced and unencumbered, and that the Society's policies on disclosure of conflicts of interest are implemented.

back to top

Obligations of Reviewers

The critical and confidential review of manuscripts is an essential element of research publications. Every scientist has an obligation to contribute to the peer review process by serving as a reviewer. Among the obligations of reviewers is the commitment to providing an expert, critical, and constructive scientific and literary appraisal of research reports in their fields of knowledge, skills, and experience in a fair and unbiased manner. In order to facilitate the prompt sharing of scientific results, it is also the obligation of each reviewer to complete their assignments promptly, within the editor's deadline. Should a delay in their review occur, the reviewer has the obligation to notify the editor at once. Reviewers should not review a manuscript if: 1) they do not think that they are competent to assess the research described, 2) they believe there is a conflict of interest or personal or professional relationship with the author(s) that might bias their assessment of the manuscript, or (3) there is any other situation that could bias their review. Employment at the same institution as one of the authors does not automatically represent a conflict. Having previously reviewed the article for another journal does not disqualify a reviewer, although the editor should be informed so the reviewer's perspective can be considered. In circumstances when reviewers need to recuse themselves, they should notify

the editor promptly, preferably with an explanation. If reviewers are uncertain whether they should recuse themselves, they should consult with the editor.

The reviewer should strive to provide accurate, detailed, and constructive criticisms, and the review should be supported by appropriate references, especially if unfavorable. The reviewer should also note whether the work of others is properly cited. If the reviewer notes any substantial resemblance of the manuscript being reviewed to a published paper or to a manuscript submitted at the same time to another journal, they should promptly report this to the editor.

No part of the manuscript under review should ordinarily be revealed to another individual without the permission of the editor. If a reviewer consults a colleague on a particular point, this fact, and the name of the collaborator or consultant, should be reported to the editor, preferably in advance. With these exceptions, a reviewer must obtain through the editor written permission from the authors to use or disclose any of the unpublished content of a manuscript under review.

back to top

Experimental Subjects

To be considered, all clinical investigations described in submitted manuscripts must have been conducted in accordance with the guidelines in The **Declaration of Helsinki** and must have been formally approved by the appropriate institutional review committees or its equivalent. All manuscripts must indicate that IRB approval was acquired; and that when informed consent was required by the IRB, that this was obtained from subjects in experiments involving humans. Investigators must disclose potential conflict of interest to study participants and should indicate in the manuscript that they have done so. The study populations should be described in detail. In many studies details of age, race, and sex are important. However, subjects must be identified only by number or letter, not by initials or names. Photographs of patients' faces should be included only if scientifically relevant. Authors must obtain written consent from the patient for use of such photographs. For further details, see the Ethical Guidelines.

back to top

Experimental Animals

A statement confirming that all animal experimentation described in the submitted manuscript was conducted in accord with accepted standards of humane animal care, as outlined in the Ethical Guidelines, should be included in the manuscript.

back to top

Clinical Trials Registration

For clinical trial reports to be considered for publication in the Journal, the Endocrine Society requires their prospective registration, as endorsed by the International Conference of Medical Journal Editors. We recommend use of www.clinicaltrials.gov. The Society's full Position Statement on Clinical Trials Registration is at the following web site:http://jcem.endojournals.org/site/misc/ClinicalTrials.pdf. All trials beginning after January 1, 2007 must have been prospectively registered before enrollment of the first subject. All trials begun before that date must be retroactively registered before submission. Please note that the Clinical Trial Registration number should be provided clearly on the title page of the manuscript.

back to top

Genetic and Genome-Wide Association Studies

To ensure rigor in genetic and genome-wide association studies and permit readers to assess their biological and clinical significance, submitted manuscripts describing such work should generally conform to the following study design criteria, which will be applied by the Journal's reviewers and editors in their evaluations.

Sample Size and Multiple Testing: Studies should include sufficient samples to have the power to detect an effect. In addition, since multiple hypotheses are often tested (*e.g.*, multiple SNPs, substratification, and multiple phenotypes), analyses and interpretations should account for the influence of such multiple testing on the findings' biological and clinical significance.

Validation Samples: The most rigorous association studies should include both a testing (or training) sample set and an independent validation series.

Functional Data: Functional data strengthen association data if the functional assay(s) have demonstrable relevance to the associated phenotype. In some instances, association studies with a single testing sample set and highly relevant functional data may be acceptable without an independent validation series.

Single Genetic Marker (e.g., SNP) versus Whole Gene/Genome Studies: Single SNP studies are acceptable when the particular SNP has strong prior claims for involvement in the phenotype of interest. However, it is desirable to examine genetic variation at least across and flanking the gene of interest when this is feasible.

Negative Association Studies: Well-designed and executed association studies that demonstrate significant negative findings will be considered if the gene in question has clear relevance to disease pathogenesis or has been implicated in prior published association studies.

back to top

Microarray Expression Studies

Genome-wide expression studies require both technical validation and an independent validation series. Technical validation entails application of a different technique (*e.g.*, RT-PCR of single genes or immunohistochemistry) to confirm the differential expression detected by genome-wide expression. An independent validation series of samples should be utilized to confirm the differential expression noted by genome-wide analysis of the initial testing sample set.

back to top

Nomenclature and Technical Requirements

The value of study data is enhanced if, where relevant, manuscripts:

- Use standard terminology for variants, providing rs numbers for all variants reported. These can be easily derived for novel variants uncovered by the study. Where rs numbers are provided, the details of the assay (primer sequences, PCR conditions, etc.) should be described very concisely.
- Describe measures taken to ensure genotyping accuracy, *e.g.*, percentage of genotype calls, number of duplicate samples that were genotyped, and percentage concordance.
- Provide approved GDB/HUGO approved gene names, in the appropriate cases and italics.
- Provide linkage disequilibrium (LD) relationships between typed variants.
- Provide information and a discussion of departures from Hardy-Weinberg equilibrium (HWE). The calculation of HWE may help uncover genotyping errors and impact on downstream analytical methods that assume HWE.
- Provide raw genotype frequencies in addition to allele frequencies. It is also desirable to provide haplotype frequencies.
- Provide the criteria they have used to select tagSNPs.
- Denote the boundaries considered when studying SNPs within a gene of interest. For example, "gene X and 100 kb upstream of the first translational start site and 150 kb downstream of the stop codon."

back to top

Manuscripts Reporting New Amino Acid or Nucleotide Sequence

Manuscripts reporting amino acid or nucleotide sequences of proteins with sequences already known from other tissues or species will be considered only if they provide new biological insight. Manuscripts dealing with partial sequence data are not likely to be considered. The Endocrine Society has established policy that deals with submission of new protein or nucleic acid sequences. When a manuscript is accepted that contains novel sequences, such sequences must be deposited in the appropriate database (such as GenBank) and an accession number obtained before the manuscript is sent to the printer. It is recommended that the following statement containing the assigned accession number be inserted as a footnote: "These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number UI2345."

back to top

Standards for Steroid Nomenclature

The 3 major classes of mammalian sex hormones - androgens, estrogens, and progestins (or progestagens or gestagens) - are defined by their biological activities, which are mediated via the well-defined androgen, estrogen and progesterone (or progestin) receptors. The principal bioactive sex steroid and natural ligand for each class is testosterone (or 5α-dihydrotestosterone), estradiol and progesterone, respectively. Androgen(s), estrogen(s) and progestin(s) are classes of compounds with hormonal activity, and not the names of individual steroids. Synthetic steroids or extracts can be considered as members of a generic steroid class (androgens, estrogens, progestins), but are distinct from the natural cognate ligand itself. Synthetic hormones or extracts of biological origin of each class may also have agonist, antagonist or mixed bioactivity in one or more classes. Therefore, the terms androgens, estrogens and progestins (or progestagens or gestagens) should be used when referring to the class of hormones, whereas when a specific natural or synthetic steroid is being used or assayed, the particular compound must be specified.

Apart from accepted trivial names, steroids should be named according to the systematic nomenclature of the IUPAC convention on Nomenclature of Steroids (Moss et al Pure & Applied Chemistry 61:1783-1822, 1989) at first mention in a single footnote defining all letter abbreviations. Subsequently, generic or trivial names or letter abbreviations, but not trade-names, should be used.

Examples of accepted trivial names include: cholesterol, estrone, 17α and 17β estradiol (estradiol is also acceptably used as the trivial name for 17β estradiol), estriol, aldosterone, androsterone, etiocholanolone, dehydroepiandrosterone, testosterone, 5α dihydrotestosterone, 5β dihydrotestosterone, androstenedione, pregnenolone, progesterone, corticosterone, deoxycorticosterone, cortisone, and cortisol.

Trivial names may be modified by prefixes or suffixes indicating substituents (as in 17-hydroxyprogesterone for 17-hydroxy-4-pregnene-3,20-dione), double bonds (as in 7-dehydrocholesterol for 5,7-cholestadien-3-ol) and epimeric configurations of functional groups provided the locus of epimerization is indicated (as in 11-epicortisol for 11α21-trihydroxypregn-4-en-3-one).

back to top

Manuscripts Reporting Novel Compounds

Manuscripts describing experiments with new compounds must provide their chemical structures. For known compounds, the source and/or literature reference to the chemical structure and characterization must be provided.

back to top

Validation of Data and Statistical Analysis

Assay validation: Bioassay and radioimmunoassay potency estimates should be accompanied by an appropriate measure of the precision of these estimates. For bioassays, these usually will be the standard deviation, standard error of the mean, coefficient of variation, or 95% confidence limits. For both bioassays and radioimmunoassays, it is necessary to include data relating to within-assay and between-assay variability. If all relevant comparisons are made within the same assay, the latter may be omitted. Authors should be aware that the precision of a measurement depends upon its position on the dose-response curve.

In presenting results for new assays, it is necessary to include data on the following: 1) within-assay variability; 2) between-assay variability; 3) slope of the dose-response curve; 4) mid-range of the assay; 5) least-detectable concentration (concentration resulting in a response two standard deviations away from the zero dose response); 6) data on specificity; 7) data on parallelism of standard and unknown and on recovery; and 8) comparison with an independent method for assay of the compound. When radioimmunoassay kits are utilized or hormone measurements are conducted in other than the authors' laboratories and the assay is central to the study, data regarding performance characteristics should be included.

Pulse analysis: Data from studies of pulsatile hormone secretion should be analyzed using a validated, objective pulse detection algorithm. The algorithm used should require that false-positive rates of pulse detection be defined in relation to the measurement error of the data set being analyzed, and the methods used to determine the measurement error should be described. The author(s) also

should describe the methods used: 1) to deal with missing or undetectable values; 2) to determine peak frequency, interpeak interval, and pulse amplitude; and 3) for statistical comparisons of peak parameters.

Data analysis: It is the author's responsibility to document that the results are reproducible and that the differences found are not due to random variation. No absolute rules can be applied, but in general quantitative data should be from no fewer than three replicate experiments. Appropriate statistical methods should be used to test the significance of differences in results. The term "significant" should not be used unless statistical analysis was performed, and the probability value used to identify significance (*e.g.*, P > 0.05) should be specified.

When several *t* tests are employed, authors should be aware that nominal probability levels no longer apply. Accordingly, the multiple*t* test, multiple range test, or similar techniques to permit simultaneous comparisons should be employed. Also, in lieu of using several *t* tests, it is often more appropriate to utilize an analysis of variance (ANOVA) to permit pooling of data, increase the number of degrees of freedom, and improve reliability of results. Authors should use appropriate nonparametric tests when the data depart substantially from a normal distribution. Analysis of variance tables should not be inserted in manuscripts. F values with the degrees of freedom as subscripts together with the *P* values are sufficient.

In presenting results of linear regression analyses, it is desirable to show 95% confidence limits. When data points are fitted with lines (as in Scatchard or Lineweaver-Burk plots), the method used for fitting (graphical, least squares, computer program) should be specified. If differences in slopes and/or axis intercepts are claimed for plotted lines, these should be supported by statistical analysis.

Authors should include in the manuscript a list of the software used for statistical analyses.

back to top

Digital Image Integrity

When preparing digital images, authors must adhere to the following guidelines as stated in the CSE's White Paper on Promoting Integrity in Scientific Journal Publications:

- No specific feature within an image may be enhanced, obscured, moved, removed, or introduced.
- Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the entire image and as long as they do not obscure, eliminate, or misrepresent any information present in the original.
- The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (e.g., dividing lines) and in the figure legend.

Deviations from these guidelines will be considered as potential ethical violations.

Note that this is an evolving issue, but these basic principles apply regardless of changes in the technical environment. Authors should be aware that they must provide original images when requested to do so by the Editor-in-Chief who may wish to clarify an uncertainty or concern.

[Please see paper of Rossner and Yamada (Journal of Cell Biology, 2004, 166:11-15), which was consulted in developing these policy issues, for additional discussion, and the CSE's White Paper on Promoting Integrity in Scientific Journal Publications, published by the Council of Science Editors, 2006.]

back to top

Publication and Production Guidelines

Proofs and Reprints

Proofs and a reprint order form are sent to the corresponding author unless the Editorial Office is advised otherwise. The author should designate by footnote on the title page of the manuscript the name and address of the person to whom reprint requests should be directed. Questions about

reprints should be directed to June Billman, Account Manager at Cenveo Publisher Services, (June.Billman@cenveo.com) (preferred), 410-943-3086 (direct) or 1-866-487-5625 (toll-free).

back to top

Publication and Color Costs

There is no submission fee for The Endocrine Society journals.

There will be a charge of $95 per page for members of The Endocrine Society and $115 per printed page for non-members. There will be a charge of $235 per color figure for members of The Endocrine Society and $735 per color figure for non-members. For more information on the benefits of membership in The Endocrine Society, please visit the Member Benefits page of The Endocrine Society's website. Authors must submit usable digital art that passes Cadmus's Rapid Inspector. Queries on page charges may be directed to June Billman at Cenveo Publisher Services, (June.Billman@cenveo.com) (preferred), 410-943-3086 (direct) or 877-705-1373 (fax).

back to top

NIH Deposits

For articles that were funded by NIH, accepted manuscripts will be submitted to PubMed Central. These manuscripts will be made freely available online twelve months after print publication. NIH will contact the author to confirm submission.

back to top

Open Choice Option

The Endocrine Society’s Open Choice program was developed to allow researcher authors the ability to provide immediate, open and free access to their work. For a growing number of our authors, providing open access is a condition of funding. For others, they simply want to have their latest findings available to the scientific domain without delay. Still others believe that paying to make their article free in the first 12 months of publication is not a worthwhile use of their grant monies.

The Endocrine Society offers authors an Open Choice option for $3,000 per article, in addition to other publication charges. Upon receipt of payment, the article will be made openly available on the journal site and the final print version will be deposited in PubMed Central for immediate public access.

Corresponding authors can indicate on the invoices included with their proofs if they wish to exercise this option. All articles will be licensed using the Creative Commons, Attribution, Non-commercial license 2.0.

back to top

Institutional Repositories and Other Archives

Authors may deposit the final PDF version of their manuscript in their institutional repository or other archive 1 year following the date of print publication. Any deposits to be made prior to 1 year following the date of print publication must be approved by the Publications Department of The Endocrine Society.