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Insights in Familial Amyloidotic Polyneuropathy Portuguese Type (Val30Met) Salivary Glands

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José Manuel Barbas do Amaral

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Monteiro FA, Sousa MM, Cardoso I, do Amaral JB, Guimarães A, Saraiva MJ. Activation of ERK1/2 MAP kinases in familial amyloidotic polyneuropathy. J Neurochem. 2006 Apr; 97(1):151-61.

Macedo B, Batista AR, do Amaral JB, Saraiva MJ. Biomarkers in the assessment of therapies for familial amyloidotic polyneuropathy. Mol Med. 2007 Nov-Dec; 13(11-12): 584-91

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Work for this thesis were performed at the following Institutions

Hospital de Santo António, Porto

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Serviço de Patologia Laboratorial
Unidade Clínica de Paramiloidose

Unidade Amilóide do Instituto de Biologia
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in memoriam of my father Sebastião

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Often then not, when we finish a job and proceed with its revision we are overtaken by feeling of omission or that something could have been expressed in other words. Notwithstanding, the thesis now under scrutiny is the result of long and arduous process that begun approximately ten years ago.

It's presented with the conviction that it may make a positive contribution to our knowledge and understanding of paramyloidosis. A highly prevalent disease in certain geographical areas of the world, patients still slowly but inexorably express organic, functional and psychological debilitating repercussions. This deeply marks physicians, allied healthcare providers, and next of kin, despite recent developments in organ transplantation and introduction of new pharmaceuticals that shed some light and awaken new hope at least for some of these sufferers.

Why did it take so long to undertake this task? Well let's just say that the path is not always as straight as it may seem and things do not always go as planned. Cross roads are encountered, decisions are to be made as to which is to be followed, taking the risk that it may lead to a dead-end. Sometimes we may simply be overloaded or just not in the mood to do our best, and impeded to continue because we are committed and obliged to provide the best care to our patients. Often these shortcomings result in misunderstanding and weakened relationships among those that willingly to help us. However, these added difficulties often lead to new uncharted, but enlightened pathways.

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Finally, to all patients, as they are the main reason why we have embraced this noble profession.

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PREFACE

PREFACE

Portugal is the largest world focus of amyloidosis associated with transthyretin mutation Val30Met. The Unidade Clínica de Paramiloidose at Hospital Santo António, in Porto, is of prime importance in the study and treatment of this disease.

I am one of the members of a multidisciplinary group organized in that Hospital and II work in the management and implementation of salivary gland biopsies for demonstration of amyloid deposits in all patients carrying the mutation (TTRVal30Met), that are proposed for liver transplantation (the only treatment available for this disease, or those who are involved in clinical trials with drugs to slow the disease progression.

In my clinical practice I observed many FAP patients in whom the diseases of the oral cavity did not seem to be more prevalent than in the general population, including tooth decay. In general there were no complaints of xerostomia or difficulties in eating, swallowing, tasting or speaking. In the FAP sample of patients studied I have not seen any patients with candidiasis or atypical dental caries in cervical, incisal surfaces or cusp tip locations.

This is the first investigation in saliva secretion rate and composition of Portuguese FAP patients. Beyond these factors we studied their oral manifestations, salivary gland histology and their relations with amyloid deposition and disease staging.

Some of the salivary parameters studied are compared with those of Johansson et al studied in Sweden FAP patients in 1992.

Histopathological studies were performed in Unidade de Neuropatologia of Hospital S. António in Porto (Director: Prof. Dr. Melo Pires) and in the Unidade Amilóide of Instituto de Biologia Molecular e Celular do Porto (Director: Prof. Dra. Maria João Saraiva) .The photographs were obtained from those study materials.

The two initial photographs of localized amyloidosis were kindly provided from the Serviço de Anatomia Patológica of Hospital Santo António (Director: Prof. Dr. Carlos Lopes).

The onset of the disease is related with the amyloid deposition which is impractical in terms of clinical practice.

Being the early diagnosis of this disease important to prevent is clinical manifestations, we tried to relate the disease evolution to some biological markers.

The deposition of amyloid in the labial salivary glands, the characterization of the saliva of FAP patients and an attempt to identify biological markers that could be useful in early diagnosis were studied in this work.

ABREVIATIONS

AA Amyloidosis	systemic amyloidosis
AGA	antigliadin antibodies
AL Amyloidosis	light-chain amyloidosis
Aβ Amyloidosis	amyloid beta protein precursor
cAMP	cyclic adenosine monophosphate
CEOTs	calcifying epithelial odontogenic tumors
DNA	deoxyribonucleic acid
ECM	extracelular matrix
ELISA	enzyme-linked immunoabsorbent assay
ER	endoplasmic reticulum
FAP	familial amyloidotic polyneuropathy
HIV	human immunodeficiency virus
Hts	salivary histatins
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL- 1β	human interleukin 1 β
IL- 2	interleukin 2
IL- 6	interleukin 6
JUNK	jun-N-terminal kinase
LSG	labial salivary glands
MAPK	mitogen activated protein kinase
MCSF	macrophage colony stimulating factor
Mg	magnesium
MG1	salivary mucin1
MG2	salivary mucin 2
MMP- 9	metalloproteinase 9
MMP- 2	metalloproteinase 3
MMP- 3	metalloproteinase 3
MMPs	metalloproteinases
Mn	manganese
mRNA	messenger RNA
MTJC7	small soluble mucins
MUC1	mucin 1 cell surface associated
MUC4	mucin 4 cell surface associated
MUC11	mucin 11
MUC12	mucin 12 cell surface associated
MUC5AC	mucin 5AC oligomeric mucus/gel forming
MUC5B	mucin 5B oligomeric mucus/gel forming
MUC6	mucin 6 oligomeric mucus/gel forming
NF- kB	nuclear factor K-light-chain-enhancer of activated B cells
NGAL	gelatinase associated lipocalin
PCR	polymerase chain reaction
Pig R	polymeric immunoglobulin
PRP 3	proline-rich protein 3
PRPs	salivary praline-rich proteins
RAGE	receptor for advanced glycation endproducts
RGD	arginine-glycine-aspartase
RNA	ribonucleic acid
s- UgA	secretory immunoglobulin A
TMT	tetramethyl benzidine

TNFα	tumor necrosis factor α
TTR	transthyretin
ZAG	zinc-alpha-2-glycoprotein

I. INTRODUCTION

1. Historical review

Familial amyloid polyneuropathy (FAP) is an inherited form of autonomic, sensory and motor neuropathy in which there is a deposition of amyloid substance in peripheral nerves and others organs.

The term "amyloid" was described in 1838 by a German botanist, Matthias Schleiden, as a normal amylaceous constituent of plants. In 1854, Rudolph Virchow used this term to describe the peculiar reaction of the corpora amylacea of the nervous system with iodine, convinced that it could be identical to starch. Virchow believed that the pale aspect of some organs (liver; spleen) in some diseases was due to the infiltration of the substance amyloid as a result of ischemia for vascular obstruction. He also noticed that the test of iodine showed reactivity with the glomeruli and afferent arteries of the kidney. He described cases with total involvement of the alimentary tract having also referred that marasmus was a prominent feature of advanced amyloid disease.

In 1856, Johann Meckel tries the iodine sulphuric acid test in different organs, such as the liver, spleen, kidneys, arteries and intestine, obtaining the blue and violet coloration in the organs that were affected by the lardaceous substance, believing that those deposits were constituted by cholesterine.

Samuel Wilks, in 1865, describes a case of primary amyloidosis in a 52 year-old man, whose organs were infiltrated by a lardaceous substance, unrelated to syphilis, osteomielite or tuberculosis. The man died from edemas and albuminuria and at autopsy, the heart was hypertrophied and the spleen was hard and with a whitish aspect. The kidneys were whitish and showed important alterations provoked by the lardaceous substance. However, this patient was uncommon because of the fact of having previous episodes of edemas for 8 years, which is much longer than the usual survival of patients with primary amyloidosis and nephrotic syndrome. He published 60 more additional cases of disease and emphasized that 5 of those cases did not have underlying disease such as syphilis, tuberculosis or bone disease. He did not agree with the term degeneration as the affected organs were filled out with a substance that would lead to the destruction of their natural elements.

Dickinson WH, in 1869, presented a case of lardaceous disease of the kidney, which was attributed to an ovarian abscess and postulated that lardaceous material was a “precipitation from the blood of fibrin, which has been dissociated from the albumin and alkali with which is normally connected”.

Weber in 1867 and Adams in 1872 reported the existence of lardaceous changes in the liver and spleen of patients with multiple spontaneous fractures and bone infiltration by that substance. These were the first reported cases of lardaceous material deposition associated with multiple myeloma.

The Real Society of London in 1871 designated a Commission, of which Wilks was a member, to attribute the definitive nomenclature of this substance. The term lardaceous was adopted which designated the whole substance of albuminous origin; the tissue contained an increase of cholesterine and 13-14% of nitrogen.

It is only with the development of new scientific technologies that this substance could be better characterized and set up the diversity of its composition.

Later, this lardaceous material started to be called amyloid and was related, in 1931 by Magnus-Levy, and in 1940 by Apitz, to Bence-Jones protein and then forms its primary nature, which was different from the one found in secondary diseases of the liver and spleen.

It is not clear why the term amyloidosis prevailed, but the reason might be related to Virchow and the use of the iodine stain as a diagnostic test (Doyle L 1988).

In 1875, André-Victor Cornil in Paris, Richard Heschl in Vienna and Rudolph Jürgens in Berlin independently reported the use of aniline dyes in the recognition of amyloid.

Paul Ehrlich in1878 used the term “methacromatic” to describe the staining reaction of amyloid. The methyl-violet was particularly better than the iodine sulphuric acid test. The methacromatic stains were replaced by Congo red, which was introduced by Bennhold H in 1922.

Congo red is an aniline dye that is used for staining textiles. It also stains all types of amyloid (Fig. 1). Congo red was used to stain tissue in 1886, but it was not until 1922 that Congo red was found to avidly bind to amyloid (Bennhold H 1922).

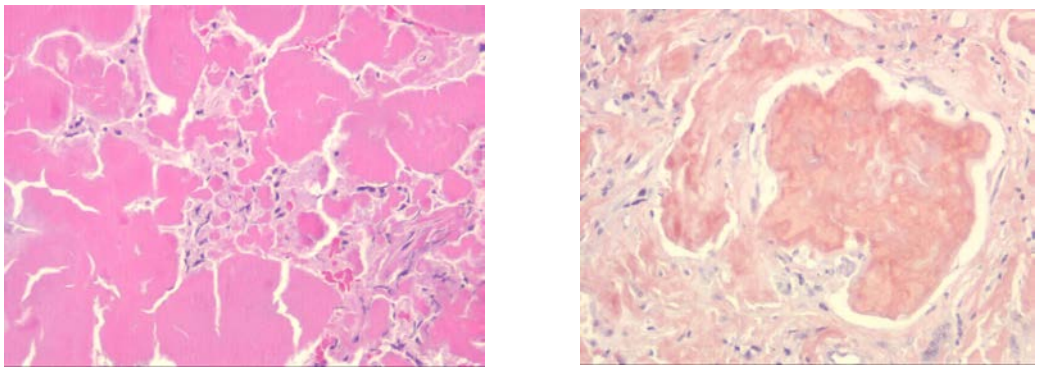


Fig. 1: Nasopharyngeal amyloid deposition, H.-E. on the left and red Congo on the right, x70

Divry and Florkin (1927) in the University of Liege described the green birefringence when an amyloid plaque from the brains of patients with Alzheimer's disease exhibited apple-green birefringence when stained with Congo red and viewed under polarized light (Fig. 2).

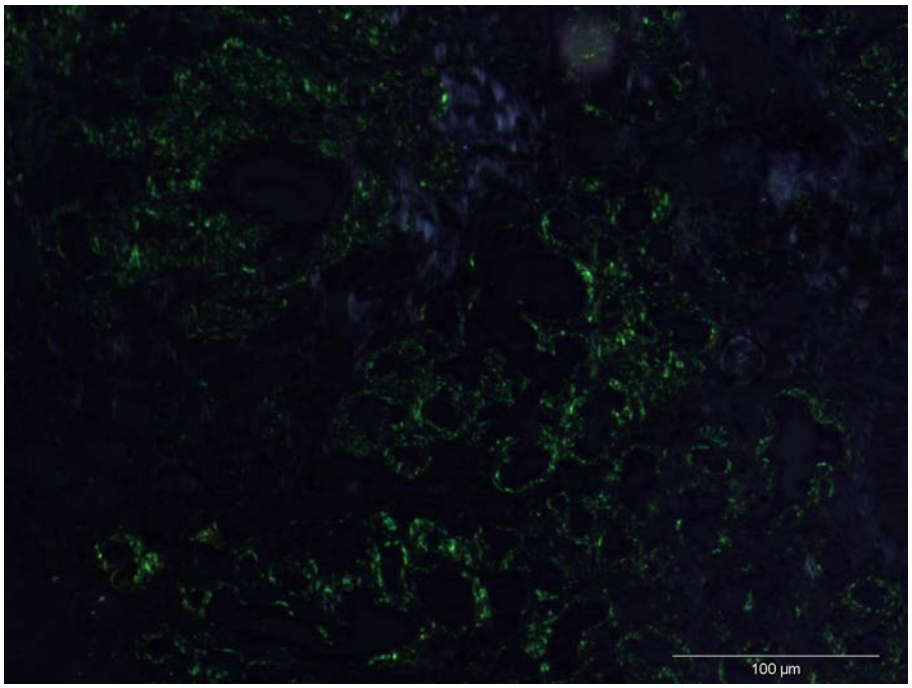


Fig. 2 - Labial salivary gland, Red Congo, Polarized light

In 1959 Cohen and Calkins first recognized that all types of amyloid demonstrated a non-branching fibrillar structure when viewed under the electron microscopy (Fig. 3).

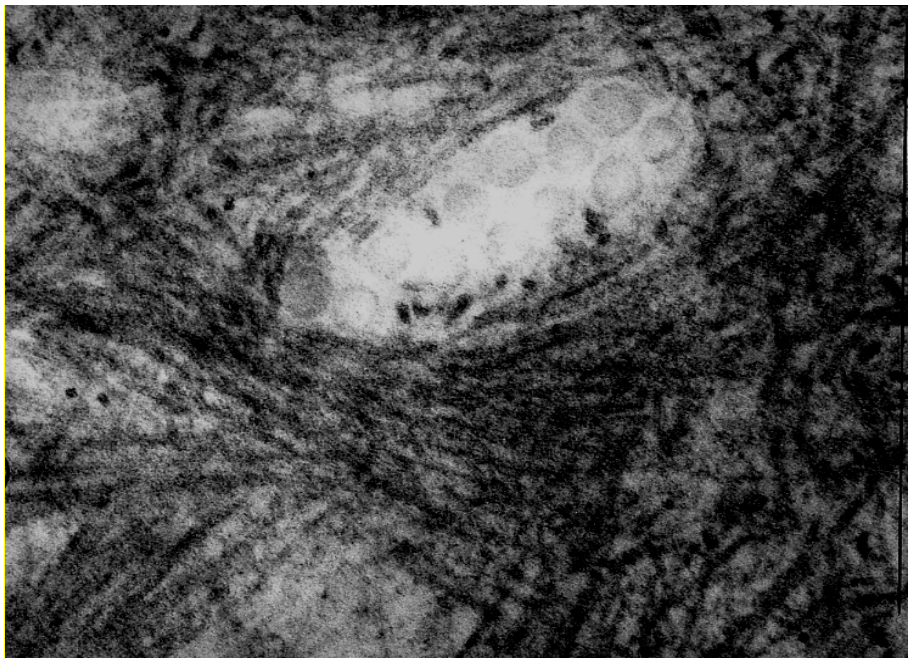


Fig. 3 - Electron microscopy: amyloid fibrils surrounding a collagen bundle, x 160.000

Eanes and Glenner, in 1968, used X-ray diffraction to study the structure of amyloid fibrils. Their results have permitted to describe this structure as being formed by the amyloid polypeptide chain with particularly characteristics.

Glenner et al in 1971 cleaved three κ-and two λ-Bence-Jones proteins from myeloma patients without amyloidosis into their variable and constant fragments. If the fragments were exposed to pepsin, precipitates of two λ-Bence-Jones proteins were formed. These precipitates stained with Congo red and presented a green birefringence with polarization microscopy.

Serum amyloid P component (SAP) scintigraphy is a technique that is been developed by Hawkins et al in 1990 for specific evaluation of Amyloidosis (Hawkins et al 1990).

In recent years it has been developed new technical devices to improve the amyloidosis diagnosis as newer echocardiographic techniques, including tissue Doppler imaging and deformation imaging (strain rate imaging and 2-dimensional speckle tracking). Using these advanced techniques, early functional impairment in cardiac amyloidosis may be detectable when the results of standard echocardiography are still normal or inconclusive (Liu D et al 2011).

To document the disease extent, it has been used the positron emission tomography imaging (Wall JS et al 2011).

Fluorescence spectroscopy (Lakowicz JR 1987), nano-electrospray ionization mass spectrometry (Mann M and Wilm M 1995), atomic force microscopy (Chamberlain AK et al 2000), circular dichroism (Kelly SM et al 2005) and laser microdissection with mass spectrometry (Cohen AD et al 2010) are among the more recent proteomic analysis to identify the potential amyloid precursors that are involved in amyloidogenesis.

2. Amyloidosis: definition and classification

2.1 Amyloid definition

Amyloid is defined as a substance of protein nature that is deposited in the extracellular spaces of several tissues (as described before). These deposits with a fibrillar structure that measure 75 to 100 Å in transversal cut and variable length (1000-2000 Å), having in X-ray diffraction studies a cross-β X-ray pattern, which is interpreted as a “pleated-sheet” structure, is formed by the amyloid polypeptide chain folding in a regular manner on itself, so that adjacent chain segments are laterally arranged in an antiparallel manner. In addition to the characteristic fibrils, a second component, the P component, has been noted in amyloid deposits. P component has been recognized by electron microscopy as a pentagonal shaped structure unit having an outside diameter of about 90 Å. On immunoelectrophoresis, it migrates as an alpha globulin and possesses antigenic identity with a constituent of normal human plasma (Serum Amyloid A).

The amyloid substance stained with Congo red had a green birefringence in the polarizing microscope. This coloration is due to the optical properties of the β-pleated structure that in contact with polarized light excludes the red component of the luminous spectrum, acquiring the typical birefringence.

The amyloid fibrils have different protein components and, for that reason, different organic and clinical manifestations (Benson MD 2001).

The distribution of amyloid deposits in the organism may be generalized with different degrees of intensity, being called systemic, or may be localized. This is a rarer form of amyloidosis, which might happen in amyloidomas, senile plaques and in the spongiform encephalopathies.

The systemic amyloidosis has specific protein components (AA in inflammatory diseases, AL related with immunoglobulins, TTR in familial forms, procalcitonin in medullar carcinoma of the thyroid, amyloid protein related with calcifying epithelial odontogenic tumor).The systemic forms can be associated to

lymphoproliferative diseases, inflammation, chronic infections dialysis, and some are hereditary diseases.

Many of the systemic amyloidosis have deposition of amyloid in several tissues (peripheral nerve, heart, cornea, etc.), which is different from localized amyloidosis, in which there are not deposition of amyloid in other organs, neither the correspondent clinical manifestations.

In the oral cavity, local amyloidosis is extremely rare. Macroglossia is the major event related to the secondary amyloidosis due to a systemic disease (Pentenero M et al 2006).

2.2. Classification

To date, there are 27 known extracellular fibril proteins in amyloid composition and at least nine of them were studied in animal models (Jean D Sipe et al 2010). Their classification is based on the nature of the protein precursor and on the distribution of the amyloid deposits, either systemic or localized.

The AA amyloidosis was formerly called secondary or reactive amyloidosis and it is a consequence of long-lasting high expression of the acute-phase amyloid protein serum AA that can be expressed and regulated through several cytokines.

AL amyloidosis contains fibrils which are derived from monoclonal immunoglobulin light chains, produced by a plasma cell clone, that proliferate normally in the bone marrow. It is usually associated with the β2-microglobulina deposition in the bones and joints (Merlini G et al 2006).

Localized specific amyloid deposits are commonly found in association with age but also with some diseases. Some well-known examples are Aβ-amyloid in the brain (associated with Alzheimer disease) and AIAPP-amyloid (in the islets of Langerhans of type 2 diabetes). Senile systemic amyloidosis is defined as a disease in which wild-type transthyretin-derived amyloid is present in the heart in an enough amount to cause cardiac dysfunction. The fibril precursors are produced close to the site of deposition.

The familial amyloidosis constitute a heterogeneous group of diseases, being the TTR amyloidosis the most frequent and presenting a predominant involvement of the peripheral nervous system (Benson MD 2001).

Other types of familial amyloidotic polyneuropathy were described as ALys (Lysozyme), AGel (Gelsolin), AApoAI (Apolipoprotein I), AApoAII (Apolipoprotein

AII), ACys (Cystatin C) and AFib (Fibrinogen α-chain). Table I presents the amyloidosis classification.

Table I. Amyloidosis classification

Amyloid protein	Precursor	Clinical manifestations
AL	Immunoglobulin light chain	Primary; Myeloma-associated
AH	Immunoglobulin heavy chain	Primary; Myeloma-associated
A β2M	β2-microglobulin	Hemodialysis-associated; joints
ATTR	Transthyretin	Familial; Senile and systemic, Tenosynovium
AA	Apo (serum) AA	Secondary, reactive
AApoAI	Apolipoprotein AI	Aorta, meniscus Familial
AApoAII	Apolipoprotein AII	Familial
AApoAIV	Apolipoprotein AIV	Sporadic, associated with aging
AGel	Gelsolin	Familial (Finnish)
ALys	Lysozyme	Familial
AFib	Fibrinogen α-chain	Familial
ACys	Cystatin C	Familial
ABri	ABriPP	Familial dementia, British
ALect2	Leukocyte chemotactic factor 2	Mainly kidney
ADan	ADan PP	Familial dementia, Danish
Aβ	Aβ protein precursor (AβPP)	Alzheimer's disease, aging
AprP	Prion protein	Spongiphorme encephalopathies
ACal	(Pro) calcitonin	C-cell thyroid tumors
AIAPP	Islet amyloid polypeptide	Islet of Langerhans Insulinomas
AANF	Atrial natriuretic factor	Cardiac atria
Apro	Prolactin	Aging pituitary Prolactinomas
AIns	Insulin	Iatrogenic
AMed	Lactadherin	Senile aortic, media
AKer	Kerato-epithelin	Cornea, familial
ALac	Lactoferrin	Cornea
AOaap	Odontogenic amelobast-associated protein	Odontogenic tumors
ASemI	Semenogelin I	Vesicula seminalis

Adapted from: Sipe JD, Benson MD, Buxbaum JN, Ikeda S, Merlini G, Saraiva MJ, Westermark P. Amyloid 2010 Sep;17/3-4):101-4.

2.3. Amyloidosis and oral cavity

The Department of Stomatology and Maxillofacial Surgery of S. António Hospital in Porto has since 1995 a multidisciplinary collaboration with the Unidade Clínica de Paramiloidose to observe and treat FAP patients. On my personal observation of about six hundred FAP patients with early disease onset there were no complaints of xerostomia or difficulties in eating, swallowing, tasting or speaking. There was no clinical evidence of amyloid deposition in the oral cavity, namely presence of macroglossia. Some edentulous patients had oral candidiasis. These observations are consistent with the published data, concerning the rarity of the oral manifestations in systemic amyloidosis.

Salivary gland parenchymal destruction and xerostomia is associated with systemic amyloidosis (von Bültzingslöwen et al 2007).

Systemic localization of amyloid lesions in the head and neck area are rare. Most cases of amyloid of the head and neck represent localized amyloid of the AL type. However, 90% of patients with systemic amyloidosis will develop amyloid deposits in the upper aerodigestive tract (Lebowitz RA and Morris L. 2003).

In localized amyloidosis, the tongue is the most frequent localization, being macroglossia the common clinical presentation. (Angiero F et al 2010).

The tongue is diffusely increased of volume, presenting the typical “scalloped tongue” type. Some patients present with multiple papules, as well as nodules with ptechia and ecchymoses that easily bleed upon a minimum traumatism. These hemorrhages are due to the deposits of amyloid substance that are localized around blood vessels. The macroglossia can interfere with speech and swallowing causing sleep obstructive apnea.

As an example of other localization, Khoury S et al in 2004 described a 73 year-old woman's case with periodontitis and dental mobility leading to an inferior molar tooth removal and curettage whose histology revealed chronic inflammation and amyloid deposits in vessel walls. Amyloidosis was characterized as secondary to rheumatoid arthritis and contributed to the aggravation of the periodontal disease.

A revision of the oral biopsies between 1992 and 2002 was performed at the Temple University (Philadelphia, Pa). In 12 patients (9 women, 3 men) the distribution of amyloid deposition was as follow: floor of mouth 4; palate 2; buccal mucosa 6; gingiva 2; tongue 3. It is not mentioned whether the lesions were obtained from patients with primary or secondary amyloidosis. These

specimens were not obtained from random tissue samples but from oral tissues where lesions were clinically evident (Stoopler ET et al 2003).

A study performed at the Emory University Hospital (Atlanta, Ga) involving 15 cases of head and neck amyloidosis between 1985 and 2005, found 15 patients (9 men, 6 women) with oral amyloid lesions distributed as following: tongue 10; larynx 3; pharynx 1; cervical adenopathy 1. The clinical types of amyloidosis included localized amyloid deposits in the larynx and tongue, plasma cell dyscrasias associated AL amyloidosis, and haemodialysis-associated amyloidosis. Secondary amyloidosis developed in one patient with carcinoid tumor (Penner CR and Muller S 2006).

In France, Paccalin M et al in 2007, in a time period between January 2001 and December 2003, involving 12 teaching hospitals and 7 general hospitals, observed 41 cases of localized amyloidosis, being included for analysis only 35 cases: 14 cases with laryngeal or sinonasal amyloidosis, 10 cases with upper respiratory tract involvement, 10 cases with genitourinary amyloid deposit, and one case with isolated colonic amyloidosis. Light chains amyloidosis was the most frequent.

A revision of the literature (Biewend ML et al 2006) found 65 publications, with a total of 290 cases of localized amyloidosis. 190 of these patients that enter a follow-up from 6 months to 23 years, only 4 patients developed systemic disease. In 91% of the cases the light chain amyloidosis was identified. In 2% of the cases amyloidosis was identified as being TTR.

3. The familial amyloidosis

De Bruyn and Stern in 1929 described a case of a 52 year-old man that complained for 3 years of pain and weakness in the limbs. He had asthenia, anorexia and profuse diarrhea. Two brothers and a sister presented with a similar clinical picture. At autopsy, microscopic examination revealed masses of a non-nucleated, homogeneous substance in the peripheral nerves. The origin of these masses appeared to be a hypertrophy of the Schwann sheets, called “plasmatic swellings”.

In 1939, Corino de Andrade observed a 37 years-old woman from Póvoa de Varzim, near Porto, Portugal, who had a peripheral neuropathy that was known as “foot disease” in the area. Thirteen years later he describes 74 patients from multiple families who presented with an insidious onset of sensorimotor

peripheral neuropathy manifested by paraesthesias or analgesia, muscle weakness and loss of reflexes. The onset of the disease was in the second or third decade of life. In addition, abdominal distension, constipation or diarrhea, loss of sphincter control and reduced libido and sexual impotency occurred. Symptoms progressed and death occurred from cachexia or infection within a decade. Biopsy of the nerves revealed amyloid (Fig. 4). This condition was referred as Familial Amyloidotic Polyneuropathy (FAP) (Andrade C 1952).

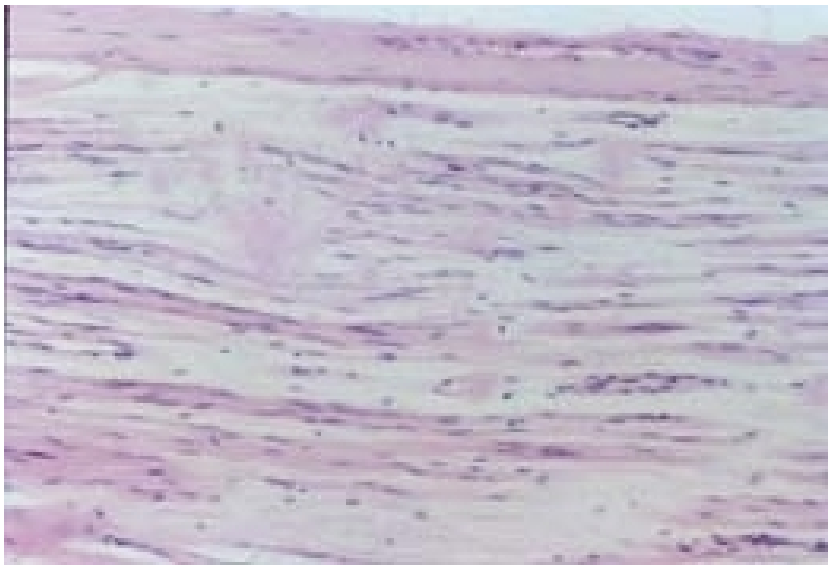


Fig. 4: Sural nerve, longitudinal section, H-E x160.

In 1965, Rune Andersson of the University of Umea in Sweden observed a 66 year-old man with a peripheral neuropathy with an evolution of 15 years. The patient was unable to stand because of severe orthostatic hypotension. The initial biopsy was negative, but a cousin that had a peripheral neuropathy and vitreous opacities, a diagnosis of amyloidosis was made (Andersson R 1970). Later he describes 16 additional cases, publishing in 1976 the clinical results of the first 60 patients with amyloidosis and polyneuropathy from northern Sweden (Andersson R 1976).

Residents of Kumamoto and Nagano municipalities in Japan have been reported with familial amyloid polyneuropathy (Tawara S et al 1981). The disease begins between 25 and 35 years of age, but the onset may occur later in life.

In Portuguese patients, Costa et al, in 1978, verified that in the immunoelectrophoresis of amyloid fibrils concentrated from the kidney, thyroid

and peripheral nerves, there was a unique protein component of FAP fibrils that was closely related with the prealbumin subunit.

Later, Portuguese investigators reported that methionine is substituted for valine at position 30 in the variant TTR (Fig. 5) (Saraiva MJ et al 1984).

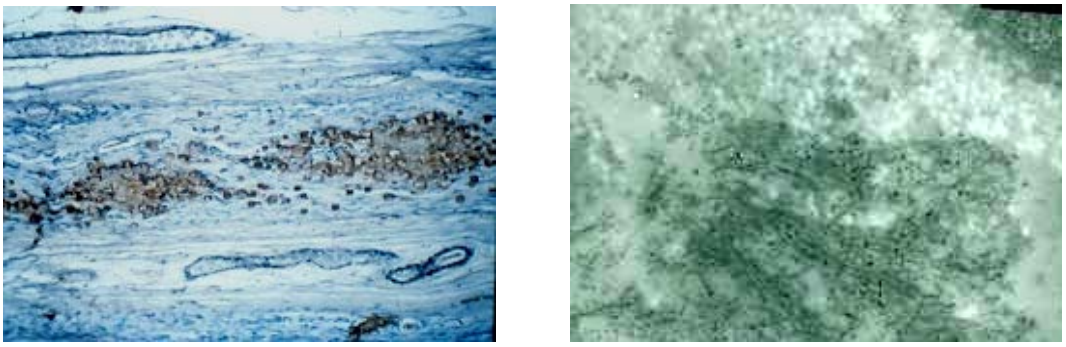


Fig 5: Immunocytochemistry using anti-bodies against TTR: on the left a longitudinal section of nerve (x 40) and on the right, in electron microscopy, an endoneurial deposit of amyloid labelled with colloidal gold particles (X20 000).

In 1932, Ostertag reported a familial amyloidotic nephropathy whose clinical picture was dominated by the arterial hypertension and mild renal insufficiency that progresses to end-stage renal failure. The amyloid deposits were provoked by a mutation in fibrinogen α-chain (Alu 554 and Glu 526) (Benson MD et al 1993; Uemichi T et al 1994).

In 1956, Rukavina J et al published the case of amyloidosis of a family of Swiss origin but resident in Indiana in the United States of America. The first manifestation was carpal tunnel syndrome followed by peripheral polyneuropathy. Cardiac involvement was not prominent and renal involvement was absent. We now are aware that it is caused by a mutation of transthyretin (SER 84) (Wallace MR et al 1988).

In 1988, familial amyloid cardiopathy was described in 5 of 12 siblings in a Danish family with amyloid congestive heart failure in the fourth or fifth decade of life. This amyloidosis was due to the substitution of the methionine for leucine in position 111(Nordlie M et al 1988).

In 1987, Benson et al published the case of a family from the area of Appalachian in the United States of America, that presented an amyloidotic cardiopathy and a carpal tunnel syndrome followed by peripheral polyneuropathy, due to the mutation of the transthyretin in that methionine, it is substituted by alanine in the position 60.

In the Dutch form of a familial cerebral amyloid angiopathy, the amyloid is composed of a β-protein identical to that found in Alzheimer's disease, except for a glutamine instead of glutamic acid at residue 22 (Luyendijk W et al 1988).

Hereditary cerebral hemorrhage with amyloidosis has been recognized in Iceland and Netherlands. In the Icelandic form, amyloid consisting of a mutant cystatin-C is found in the small arteries, meninges and brain (Olaffson I and Grubb A 2000).

4. Transthyretin

4.1. Definition

Transthyretin is a single polypeptide chain of 127 amino acid residues identified in 1974 (Kanda Y et al 1974) initially designated as pre-albumin, because protein electrophoreses migrates before the albumin. The gene that codifies its synthesis is localized in chromosome 18 (18q 11.2-q 12.1), and most of the patients are heterozygous, having a normal allele and a mutant allele. It spans approximately 7 kb and has four exons. Exon 1 codes for a signal peptide of 20 amino acid residues and only the first three residues of the mature protein; exon 2 codes for amino acid residues 4-47; exon 3, amino acid residues 48-92 and exon 4, 93-127 (Sasaky H et al 1985). Four monomers associate non covalently to form the tetrameric plasma protein, which has binding sites for thyroxin in a central channel and surface receptors for RBP/vitamin A (Blake CC et al 1974). Essentially, all plasma transthyretin is synthesized in the liver, but the protein is also synthesized in the choroids plexus of the brain and the retinal pigment epithelium of the eye (Dickson PW et al 1985).

Its plasmatic concentration varies between 20 and 40 mg/ dl. The plasmatic levels of TTR are reduced in the patients with FAP, although the levels of the retinol and thyroxine stay normal (Saraiva MJ 1983).

In 1984, TTRV30M was identified as the responsible mutation by the familial polyneuropathy amyloidotic of the Portuguese type (Saraiva MJ et al 1984). More than 100 mutations in the primary structure of the transthyretin have been discovered in association with amyloidosis ever since (Connors LH et al 2003). Many of the mutations result from the substitution of only one amino acid (Uemichi T et al 1999).

TTRV30M amyloidosis is the most common form of autosomal-dominant hereditary systemic amyloidosis and the one that presents larger geographical

distribution. Stability studies have shown a relationship between the amyloidogenic potential of TTR and a decrease in tetrameric stability (Quintas A et al 2001). Several forms of TTR mutants, as well as normal TTR, possess the capacity to form fibrillar structures (Saraiva MJ 2001).

PCR technology analysis restriction fragments of length polymorphism are commonly used in molecular diagnosis of familial amyloidotic polyneuropathy (Saraiva MJ 2001).

4.2. Amyloidogenesis

Several studies suggest that amyloidogenic mutations destabilize the native molecule of TTR inducing conformational changes, which leads to dissociation of the tetramers into partially unfolded species, which can subsequently self-assemble into amyloid fibrils (Lai Z et al 1996; Kelly JW 1997; Cardoso I et al 2002).

Under physiologic conditions including temperature, pH, ionic strength and proteins concentration, mutants TTR molecules can dissociate into native monomers with distinct compact structures capable of partially unfolding and forming high- molecular-mass soluble aggregates (Quintas A et al 2001). Studies with native TTR showed that elevation of the temperature can induce normal TTR to form amyloidogenic aggregates in physiologic conditions of pH (Chung CM et al 2001). Mutant TTR molecules have a weak thermodynamic stability (Sekijima M et al 2003).

Under high hydrostatic pressure, native TTR can undergo partial misfolding to form amyloidogenic species (Ferrão-Gonzales AD et al 2003). A hot spot for amyloidogenic mutations occur between residues 45 and 58 (Serpell LC et al 1997).

The crystal structure of L55P TTR has revealed rearrangements in strands C and D, where proline for leucine substitution disrupts the hydrogen bonds between strands D and A, destabilizing the monomer-monomer interface contacts (Lei M et al 2004). When size-exclusion chromatography was used to monitor the amyloid formation of TTR variants including L55P and V30M, a fraction of TTR monomers was detected preceding aggregation (Quintas A et al 2001). Identical observations were made in analytical ultracentrifugation studies (Lashuel HA et al 1998) and later confirmed by structural analysis of TTR fibrils (Redondo C et al 2000; Cardoso I 2002).

The role of proteolysis in the pathogeneses of amyloidosis it is not completely known. After limited proteolysis, N-terminally truncated dimmers can form amyloid fibrils (Schormann N et al 1998).

It was demonstrated the presence of non-fibrillar aggregates before the presence of amyloid fibrils in nerves of patients with FAP in initial stage of the disease (Sousa MM et al 2001a).

Amyloid fibrils contains physiologic components such as serum amyloid P component (SAP), apolipoprotein E, elements of the basal membrane and of the extra-cellular matrix, suggesting that these elements precede and are important in the protofibrillar and fibrillar formation of the amyloid deposits (Sousa MM et al 2004).

Although TTR is synthesized mainly in the liver, it is typically deposited in several tissues (Sawabe M et al 2003; Ando Y et al 2005). It is likely that the above described endogenous components may initiate TTR deposition within a tissue and that the distribution of TTR deposition reflects the presence of these components.

There are several studies that demonstrate the involvement of the glycosaminoglycans in TTR deposition. The glycosaminoglycans are a heterogeneous group of highly sulfated carbohydrates that regulate a number of important physiologic processes. They are mainly found in proteoglycans attached to a variety of core proteins, which may be membrane-bound or secreted (Small DH et al 1996). The glycosaminoglycans are found in association with amyloid deposits, including TTR amyloid (Husby G et al 1994; Inoue S et al 1998).

There is a close association between the presence of amyloid deposits and the basement membrane around myocardial cells in cardiac deposits (Sawabe M et al 2003). Studies by Smeland S et al in 1997 have shown that TTR bind to the basement membrane highly sulfated proteoglycans perlecan. Some studies suggest that glycosaminoglycans, in particular heparan sulfate, can influence amyloidogenesis in vivo. Heparan sulfate can bind to amyloid and promote fibrillogenesis. Amyloid deposition can be seen in association with basement membranes which are rich in heparan sulfate proteoglycan (Ancsin JB 2003).

In FAP, there is frequent amyloid deposits amyloid in the endoneurium (Sobue G et al 1990), which is rich in extracellular matrix proteins including chondroitin sulfate proteoglycans (Dubový P et al 2002).

This is particularly evident and precocious in the basement membranes of salivary glands and sweet glands acini and in the base of the erector pilli muscles (Guimarães A et al 1980; Gabriel CM et al 2000).

A number of studies, particularly developed for Alzheimer disease, have provided strong evidence that oligomeric or low-molecular-mass diffusible species are the most toxic forms of Aβ. In general, low-molecular-mass oligomeric or protofibrillar species of amyloid proteins seem to be much more neurotoxic than larger amyloid fibrils (Lambert MP et al 1998; Sousa MM et al 2001a; Bucciantini M et al 2002; Matsubara K et al 2005).

4.3. Neurodegeneration induced by TTR

FAP is an axonal neuropathy. Besides the axonopathy there is also advanced nervous degeneration and loss of nervous cell in sensory and autonomic ganglia. In dorsal root ganglia, there is a severe loss of neuron population with preferentially loss of small diameter neurons (Sobue G et al 1990; Toyooka K et al 1995), which correlates well with the loss of low diameter peripheral nerves fibers involved in pain and temperature sensation and autonomic function. The axonal fiber degeneration begins in the unmyelinated and low diameter myelinated fibers (Fig. 6) and only in the advanced cases are the heavy myelinated fibers affected (Dyck PJ and Lambert EH 1969; Guimarães et al 1990).

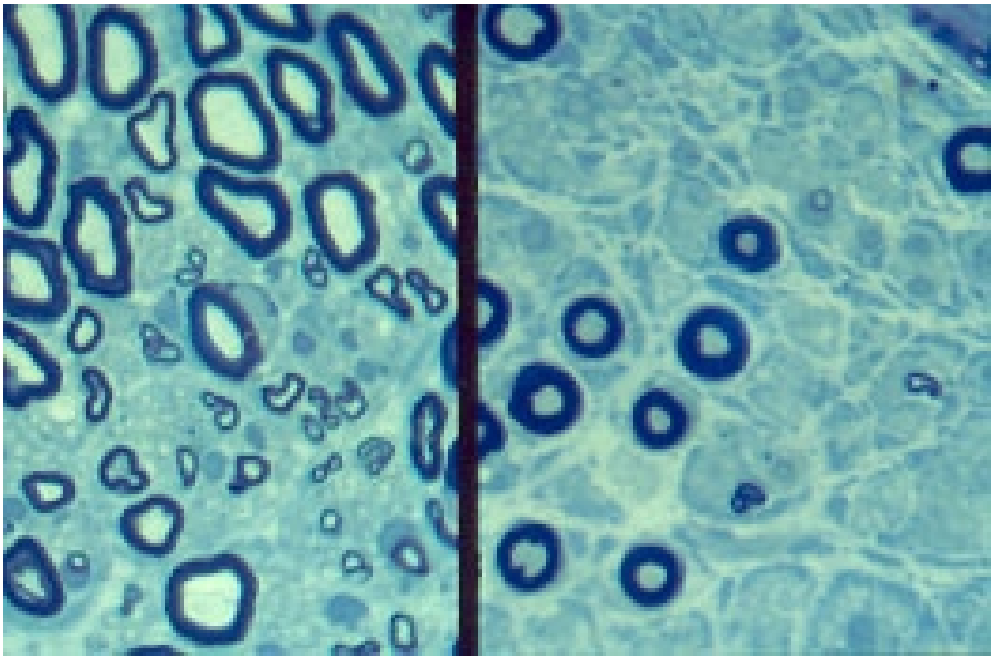


Fig. – 6 –A normal nerve on the left and a FAP nerve on the right, toluidine blue, x 1.000

In sensory and autonomic nervous system of FAP patients there is a characteristic progressive ascending neuropathy (dying-back type), as established by electroneurophysiological studies (Luis ML 1978). Histopathological analysis of 13 autopsies of typical Portuguese FAP patients, deceased at a terminal stage, as well as more than 400 nerve biopsies of FAP patients were performed and examined in Portugal (Guimarães A et al 1988). This partially published study probably represents the largest sample of FAP patients examined so far and confirmed initial loss of unmyelinated and small diameter myelinated fibers (fig. 7). Preferential loss of axons in large myelinated fibers (Misu et al 1999) and significant myelin damage (Dyck PJ and Lambert GH 1969; Sobue G et al 1990) have been described in non-Portuguese kindred; however these findings are atypical.

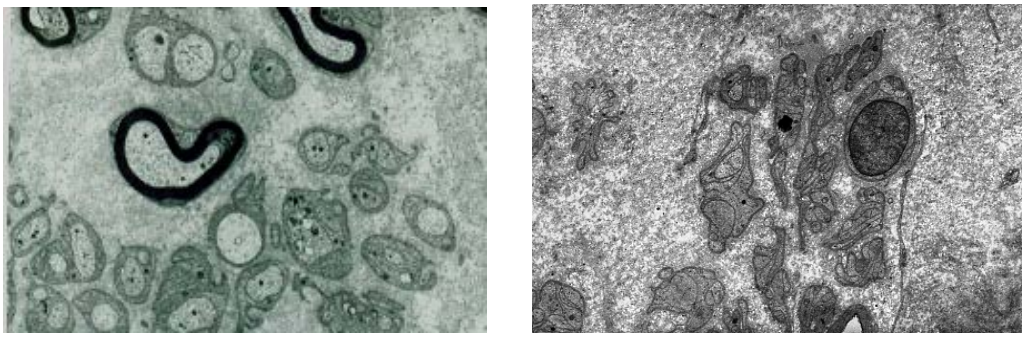


Fig. 7: Normal density of unmyelinated axons in a normal nerve and in the nerve of a late stage FAP patient. Electron microscopy x 2.800

In FAP, Schwann cells are sometimes in contact with amyloid deposits and have been described as presenting degenerative changes (Fig. 8), including disappearance of basement membrane and proliferation of distorted processes-Bungner bundles (Coimbra A and Andrade C 1971b; Araky S and Yi S 2000).

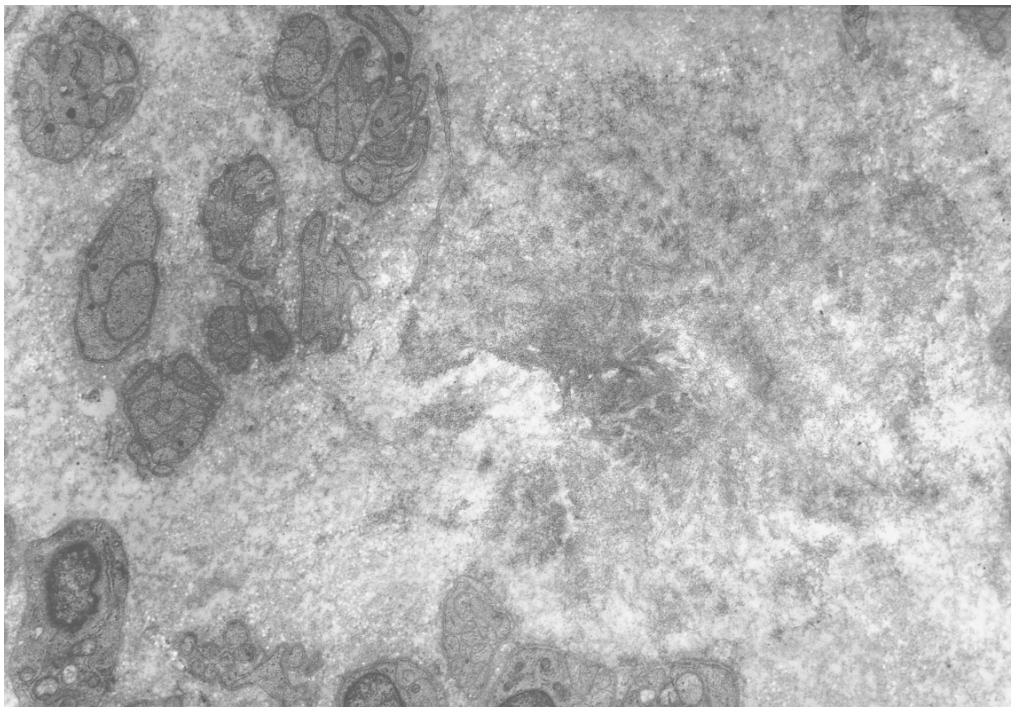


Fig. 8: Schwann cell cytoplasm, without axons, near a spheroid amyloid deposit. Electron microscopy x 8.000

Some authors postulated that amyloid physically displaces normal elements of peripheral nerves, ultimately resulting in neurons and nerve fiber loss (Fig. 9) (Dyck PJ and Lambert GH 1969; Said G et al 1984). Morphological and morphometric analysis of sural nerve biopsies of asymptomatic carriers in FAP

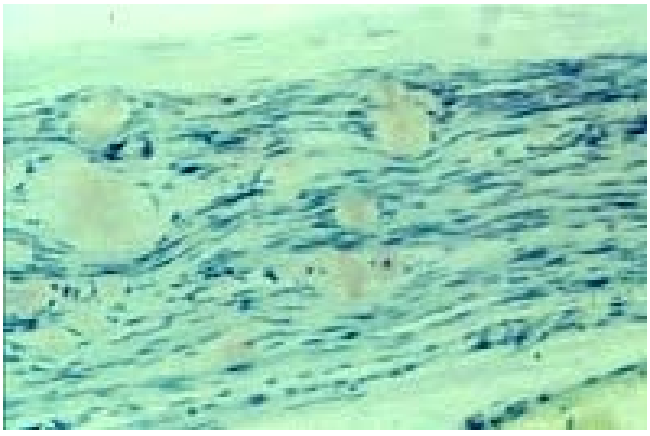


Fig. 9. Massive deposition of amyloid with a spheroid form.H-Congo red x 120

patients in different stages of the disease were carried out having as purpose to find the “starting” lesion of the disease, but the results were unconvincing,

suggesting that the answer was not in the peripheral nerve lesions (Guimarães A et al 1988; Leite I et al 1988).

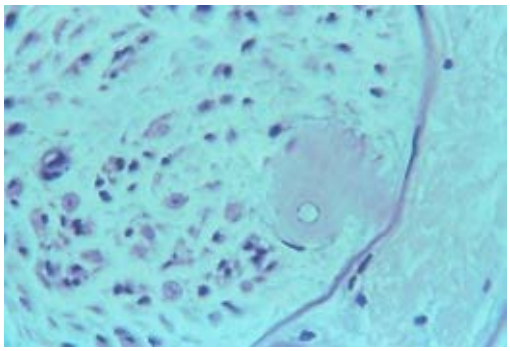


Fig10. Amyloid deposition a fibrosis endoneurial vessel wall, with a contiguous spheroid deposit. H-E x 160

Amyloid deposits are often found around blood vessels where they generally form a symmetrical annulate cuff sometimes in contact with spheroid formations (Fig. 10). However, there are also many isolated globular deposits in the endoneurium without physical relation with vessels. Despite this preferential perivascular distribution of amyloid, there is an evident fibrosis that is generalized and not patchy (Fig. 11).

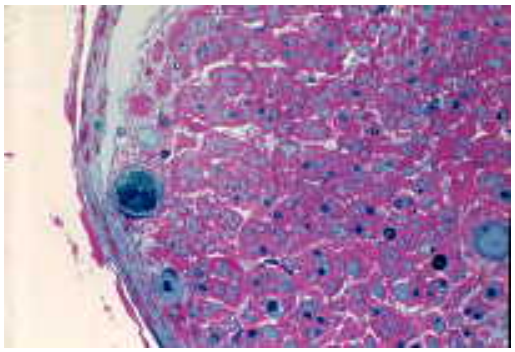


Fig 11. Severe endoneurial fibrosis in a nerve without myelinated nerve fibers. Methilen blue fucsin x 160

The lack of a cause-effect relationship between amyloid deposition and neurodegeneration in FAP suggest the importance of other non-TTR contributing factors and/or toxicity of non-fibrillar TTR aggregates. The later hypothesis has been raised even prior the identification of mutated TTR as the major constituent of amyloid fibrils. In 1971, Coimbra and Andrade suggested the possibility “that

fibril differentiation takes place within the matrix and that pre-existing collagen fibrils play some physical role in orientation and facilitating fibril formation". Reports on electron microscopy analysis of FAP nerves described not only the presence of fibrillar material, but also the coexistence of non-fibrillar aggregates, at time of unknown origin (Coimbra C Andrade A 1971a). Despite this suggestion of deposition of TTR in the form of non-fibrillar aggregates occurring locally before amyloid formation, only recently this concept was demonstrated (Sousa MM et al 2001a).

Local cellular activation resulting in cell dysfunction and death may contribute to the pathogenesis of amyloid-related disorders. Pro-inflammatory cytokines mainly, TNFα, IL-1β and MCSF are upregulated in FAP nerves due to the early presence of non-fibrillar aggregates (Sousa MM et al 2001a, b).

Oxidative stress is implicated in generation of reactive oxygen and nitrogen species and has been widely implicated in the regulation of neuronal proliferation, survival and differentiation pathogenesis of both normal aging and neurodegenerative disorders (Contestabile A and Ciani E 2004). The involvement of oxidative stress was first examined on human colon biopsy samples trough the immunohistochemical use of two markers of lipid peroxidation (Ando et al 1997) Data relating TTR deposition, degeneration and oxidative stress in FAP nerves, was lacking, until FAP nerves were probed for nitric oxide synthase antigen and increased levels were observed especially on the axons (Sousa MM et al 2001b).

Apoptosis is a common mechanism of cell death in neurodegenerative disorders (Agostini M et al 2011). In the case of FAP, local expression of cytokines and nitric oxide synthase in neurons suggested that programmed cell death might be triggered. Supporting the concept of toxicity of non-fibrillar TTR found by immunohistochemistry for proinflammatory and oxidative stress-related molecules, it has been shown that caspase-3 activation occur in vitro in a Schwann cell line when exposed to TTR aggregates; soluble TTR and longer fibrils did not active caspase-3 (Sousa MM et al 2001a).

Autonomic disturbance involving the gastrointestinal tract and the cardiac conduction system are characteristic of FAP. In both systems, degeneration has been examined and correlated with amyloid deposition and clinical symptoms. In the upper gastrointestinal tract, hypomobility resulting from both amyloid depositions in the stomach and degeneration of the intrinsic autonomic nerves was demonstrated, being hypothesized that it may be responsible for anorexia, nausea and vomiting (Ikeda S et al 1982).

A high incidence of cardiac conduction disturbances is a salient feature in FAP and amyloid infiltration of the conduction system has been found (Wiklund U et al 2008). Most conduction abnormalities are irreversible, indicating amyloid infiltration as the predominant pathophysiologic mechanism. Furthermore, patients with FAP have sympathetic cardiac denervation despite a preserved ventricular systolic function (Delahaye N et al 1999).

Pathogenic forms of amyloidogenic molecules might perturb cellular properties by engaging cellular receptors. Receptors for advanced glycation endproducts (RAGE) are a member of the immunoglobulin superfamily with a broad repertoire of ligands, among which several amyloid-associated macromolecules (Bucciarelli et al 2002). In the nerve, glomeruli and gastrointestinal tract, the distribution of RAGE correlates with TTR deposits (Sousa MM et al 2000; Matsunaga N et al 2002).

RAGE plays an important role in a variety of physiological events and regulates nuclear factor k-B, mitogen activated protein kinase (MAPK), and Jun-N-terminal kinase (JUNK) signaling (Ding Q et al 2005), all of which may be affected in FAP *in vivo* (Monteiro FA et al 2006). However, it is unclear whether all of the neurotoxic effects could be mediated through a single receptor. The susceptibility of cells to amyloid toxicity is related to the capacity of the cells to buffer the intracellular calcium concentration, suggesting that disruption of calcium homeostasis may be a key event in amyloid toxicity (Cecchi C et al 2005). In support of this idea, recent studies showed that TTR may cause endoplasmic reticulum (ER) stress, resulting in the release of calcium from ER stores (Teixeira PF et al 2006).

Hou X et al in 2005, in an attempt to identify the receptor responsible for the toxic effect of TTR, examined the binding of TTR to a plasma-membrane-enriched fraction isolated from neuroblastoma cells. In agreement with Cecchi et al in 2005, they found that the binding of TTR to the membrane and the extent of disruption of membrane fluidity correlated with the degree of toxicity. They showed that TTR aggregates induce calcium influx in the same cell type. As calcium channels are localized to specific lipid raft domains within membranes (O'Connell KM at al 2004) and as disruption of these domains has been shown to activate voltage-gated channels (Davies A et al 2006), this raises the possibility that TTR-mediated disruption of lipid raft organization may lead to calcium entry (Hou X et al 2007).

It is clear that what is learned from the study of one amyloidosis may have application to another amyloidosis. Although most studies have focused on the effects of one, or perhaps two amyloidogenic peptides or proteins, it can be argued that a more integrated approach to the study of amyloid neurotoxicity is needed.

5. Familial amyloidotic polyneuropathy V30M

Corino Andrade's paper published on Brain in 1952 entitled "A peculiar form of peripheral neuropathy-familial atypical generalized amyloidosis with special involvement of the peripheral nerves". Although his clinical description stays updated, the progress of the biochemistry and molecular biology has been important for the establishment of transmission way, in order to understand epidemiological aspects and different forms of clinical presentation.

5.1 Geographical distribution

Portugal represents the largest world focus of the disease that is also present tin Sweden (Sousa A et al 1993), Japan (Ikeda S et al 2002), Balearic Islands-Spain (Munar-Qués M et al 2005), Italy (Saraiva MJ et al 1988), United States of America (Benson MD et al 1985), Brazil (Palácios SA et al 1999) and other countries. In Portugal the penetrance is elevated (80%) and the symptoms typically begin before the 40 years of age (Sousa A et al 1995), having been identified some families with later onset (Coutinho P et al 1980; Coelho T et al 1994; Conceição I and Carvalho M 2007). Some families have a more precocious onset of the disease and develop it with more gravity (Sousa A et al 1995).

Distribution of haplotypes associated with V30M disease in various populations has been established, being not consistent with genetic drift arising from one ancient founder (Yoshioka K et al 1989; Li S et al 1993; Waits RP et al 1995).

In Portugal and Sweden only haplotype I had previously been found associated with the mutation (Soares ML. et al 2004). The second most common haplotype III segregates with V30M is found in French, Italian, British and Japanese kindred. Thus, the evidence for a recurring mutation is strong and the hypothesis of a single founder could only be explained by a rare combination of events (Soares ML et al 2005).

5.2. Inheritance and age of the disease onset

In Unidade Clínica de Paramiloidose in Porto registries of about 300 families and more than 3000 carriers exist. Being the disease autosomal-dominant, it was expected the same number of men and women to be affected. Observations of Corino of Andrade revealed preponderance marked for the masculine sex, confirmed by subsequent studies (Ribeiro R et al 1961). The ratio man / woman are of 1.3/1 (Sousa A 1995).

Most of the patients presented with the disease onset on average at 35 years of age. There is an influence of the progenitor transmitter in the time of clinical presentations, getting sick early, children or mothers affected earlier (Sousa A 1995). As penetrance is more or less completed, the beginning is early or later, having cases in which the disease had a long evolution.

Swedish carriers of TTR V30M display lower penetrance (approximately 5%) and later onset, with symptoms usually developing after 50 years (Andersson R et al 1976; Sousa A et al 1993). Disease onset in Japanese patients may be early or late (Tashima K et al 1995). The Portuguese and Japanese kindred present a great variability concerning the symptoms onset (Sequeiros J and Saraiva MJ 1987; Ikeda S et al 2002). In the two populations the first symptoms classically begin before the 40 years of age, what is known as early onset. However, in the last years many carriers begin the disease in later ages (Sequeiros J and Saraiva MJ 1987; Coelho T et al 1994).

The age of the disease onset in the patients of Balearic Islands is larger than the Portuguese patients (Sousa et al 1995), or the Japanese (Ikeda S et al 2002), and Brazilian (Saporta MA et al 2009), but inferior to Swedish patients (Sousa A 1995; Bonaïti et al 2010).

The cases with late onset had a different geographical distribution, but the clinical presentation is similar to the one observed in cases at the early onset (Coelho T et al 1994).

A recent study carried out in the Department of Neurology of the Faculty of Medicine of the University of Lisbon (Conceição I and Carvalho M 2007) involving 78 different families, verified that the patients observed had a later onset of disease than patients of north Portugal where FAP is more prevalent and with a distribution similar of cases of early and late onset of disease. Only three families were originally from endemic areas in the north of Portugal where the disease was described initially, and all of them presented the classical onset of clinical

presentation. Therefore, other factors, either genetic or environmental, that are unknown may be important in the phenotypic expression of the disease.

5.3. Clinical manifestations

In hereditary diseases of late clinical expression like FAP, with an insidious and subjective onset, it is practically impossible to establish the beginning of the symptoms and therefore to validate the clinical variability.

Due to the dominant characteristic of the genetic transmission, the affected individuals have a perfect knowledge of the symptoms before even feeling them, by the fact that they already see the beginning and development of the disease in their parents and uncles.

The disease is highly disabling and fatal, on average 11 years after onset unless hepatic transplant is performed (Sousa A. et al 1995). The description of Corino of Andrade constitutes the main reference of neuropathy description (Andrade C. 1952). A revision of 483 cases of FAP contributes to a better understanding of the neuropathy evolution (Coutinho P et al 1980).

As the neurophysiologic and histopathological evaluation by itself is insufficient for the disease staging, a semi-quantitative scale that includes the weight, the autonomic, sensitive and motor neuropathy was adopted (Macedo E et al 1988).

Involvement of other organs, namely the heart, eye, kidney, endocrine and vesico-sphincterian function allowed to establish differences among the clinical manifestations attributable to the mutation V30M and other mutations of TTR.

5.4. Sensitive and motor neuropathy

Sensory symptoms are usually the first evidence of neuropathy. The clinical onset includes distal paraesthesias, numbness, sometimes in association with burning or pseudoradicular pain. Almost simultaneously with distal paraesthesias, the first objective alterations of the sensitivity appear (Andrade C. 1952). The alterations of the superficial and deep sensitivity had an ascending progression reaching the proximal extremities of the lower limbs and posteriorly involving the hands, with a centripetal progression and attaining the anterior portion of the trunk (apron distribution).

The motor alterations appear later and begin at the extensor muscles of the foot. The tendinous reflexes are abolished and the muscular atrophy is evident. In the lower limbs the combination of alterations of the sensitivity and mobility causes a “steppage type” typical march (Ribeiro RM et al 1961).

The trophic lesions appear relatively later and range from atrophy of the skin into feet ulcers and bone necrosis (Charcot arthropathy) with mutilation of the extremities. Besides, with these manifestations it is frequent to find lesions that result from the thermal and painful hypoesthesia, as for instance, burns and traumatic lesions, many of them secondarily infected.

5.5. Autonomic neuropathy

The manifestations of the autonomic nervous system can precede the onset of the sensitive and motor neuropathy (Ducla S et al 1994). This is explained by the early loss of unmyelinated nervous fibers that are the organic support of the function of the more distal part of the autonomic nervous system. This loss is confirmed by histology of nerve biopsies that confirm a predominant decrease of the small unmyelinated fibers in comparison to myelinated fibers (Yamamura et al 1988, Guimarães et al 1988). The progressive denervation of several organs can contribute to the most frequent clinical manifestations, such as gastrointestinal, glandular, vesical, sexual and cardiovascular (Dyck PJ et al 1969; Guimarães A et al 1980; Takahashi et al 1988).

5.6. Gastrointestinal manifestations

The gastrointestinal manifestations constitute one of the most relevant aspects of FAP clinical picture. Frequency and intensity of the symptoms can have an important negative influence in the well-being of the patients. Constipation is the earliest symptom, quickly alternating with diarrhea, being this one persistent later. Degeneration of the intrinsic autonomic nerves was demonstrated and may be the cause for morning nauseas, pre-prandial vomits and dysphagia due to gastric retention (Ikeda S et al 1982). Marked alterations of the mobility are the main cause of the digestive manifestations (Saraiva MN 1999). These changes may contribute to the low index of corporal mass in these patients, which was demonstrated to be a reliable prognostic factor (Suhr O et al 1994).

5.7. Cardiovascular manifestations

A high incidence of cardiac conduction disturbances is a salient feature in these patients, expressed as cardiac dysrhythmia, syncope and sudden death. These alterations are due to the sympathetic and parasympathetic involvement that may be precocious in the disease course. Conduction disturbances are irreversible and may have a contribution of an abundant amyloid infiltration of the cardiac conduction system. Heart failure is not frequently seen in Portuguese patients, being diastolic function the first change observed. Only in later disease staging there is cardiac ventricular hypertrophy and systolic malfunction occurs (Fonseca C et al 1997). The presence of orthostatic hypotension can be severe. Modification of circadian rhythm of arterial pressure by its inversion with nocturnal arterial hypertension is another cardiovascular manifestation seen in these patients (Freitas AF 1986).

5. 8 Endocrine manifestations

Deposition of amyloid substance in the pancreas leads to alterations of the glucose tolerance, but with normal levels of glycaemia at fasting (Hofer P-Å and Andersson R 1975). With the administration of an overload dose of glucose, an abnormal answer of glycaemia, glucagon and insulin, with transitory hypoglycemia and hyperinsulinemia is obtained (Ando Y et al1991).

Deposition of amyloid substance in thyroid and ovary, being very abundant, does not usually provoke significant functional alterations of these organs (J Pereira Guedes1976).

5.9. Sexual and vesico-sphincterian disturbances

Initially the libido, orgasm and ejaculation are preserved. Erectile dysfunction is one of the most constant and earlier symptoms of FAP. Sometimes interpreted as psychosomatic origin, only the emergence of other symptoms and the family history allows interpreting this manifestation (Alves M et al 1997).

Female patients had a significantly higher number of children and the length of their reproductive period was also greater than that of men (Sousa A et al 1995).

There is a loss of sensation of vesical fullness and a growing of residual urine volume due to the incapacity of muscular contraction. It increases the capacity of the bladder and there is an increase of the intra-vesical pressure. The patients are socially limited and fifty percent of the patients had in some phase of

evolution of the disease symptomatic or asymptomatic urinary infections (Andrade MJ 2009).

5.10. Renal manifestations

Although amyloid deposition is frequent and abundant in glomeruli and renal medulla, the nephropathy in FAP occurs in only one third of patients. The progression of chronic renal insufficiency appears in only 12%. Independent risk factors for nephropathies presentation are the feminine sex, a later onset of the neurological disease and a familial history of renal disease. Microalbuminuria may be in some patients the first clinical stage of nephropathy (Lobato L 2003).

5.11. Cutaneous manifestations

Xerose, seborrheic dermatitis, traumatic lesions and burns, acne, perforating ulcers and onychomycosis are seen at the most advanced disease staging (Rocha N et al 2005). There is early amyloid deposition around the acini of sudoriparous glands and of arretori pili muscles, and as a result, skin biopsy is frequently used as a diagnostic tool.

5.12. Glandular manifestations

The most frequent clinical manifestations are linked to malfunction of exocrine glands, such as salivary glands (xerostomia), the lachrymal glands (xeroftalmia) sudoriparous glands (xerose) and several glands of the alimentary tract (bowel habits disturbances and mal-absorption syndrome).

Histopathology of labial salivary glands, one of the main objectives of this work, will be described later (Fig. 12).

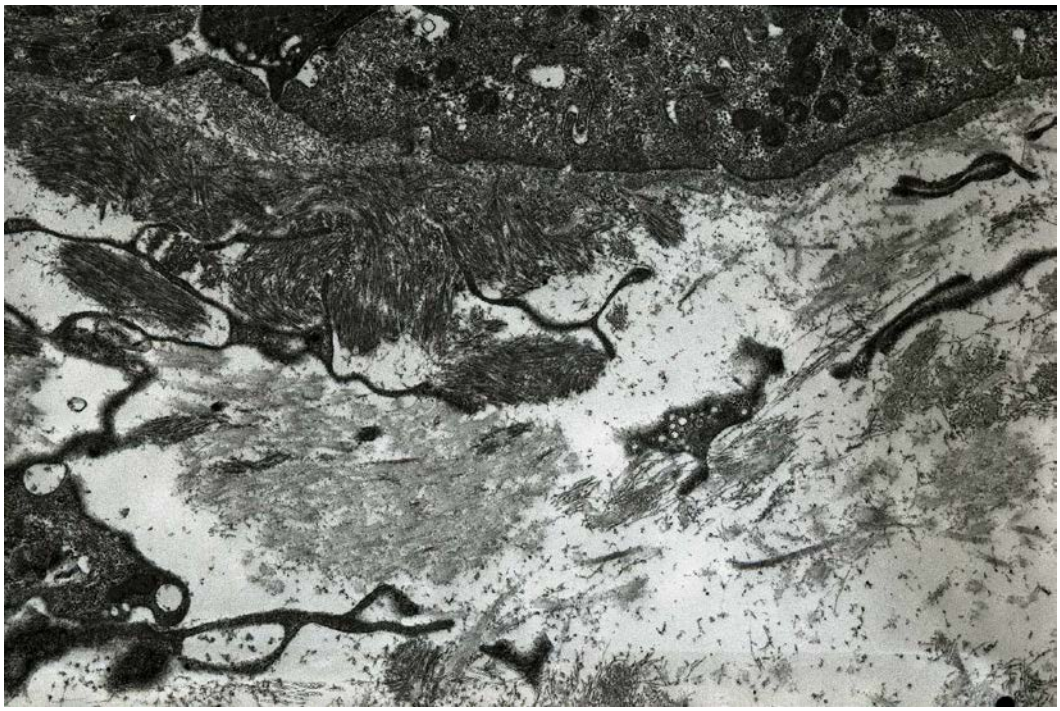


Fig. 12: Amyloid deposition near the basal membrane of a labial salivary gland. Electron microscopy x 16.000

5.13. Other manifestations

FAP patients presented a major risk to develop osteoporosis, even in early disease staging. Disease duration increases the risk of osteoporosis that can either be attributed to dysautonomia, or to the low index of corporal mass that these patients present at the most advanced of the disease staging (Conceição I et al 2005).

Normochromic or normocytic anemia is frequently seen in patients with preserved renal function. Deficit of serum iron and of erythropoietin are also seen (Beirão I et al 2004).

6. Salivary glands

There are three pairs of major salivary gland on the face: parotid, submaxilar and sublingual glands. Additionally, there are numerous minor salivary glands located in the palate, oral mucosa, tongue and lips that contribute to salivary outflow. In this section, we review the embryogenesis, histology, ultrastructure of ductal and acinar system that produces saliva, the composition of the connective tissue and vascularization and nerve supply.

6.1. Embryogenesis

Salivary glands have origin in the ectoderm of the oral cavity and, for that reason, they are comparable to the others ectodermic derived tissues, such as sudoriparous, sebaceous and mammary glands. Salivary glands arise as solid epithelial buttons of the oral mucosa with variable growth potential. The emergence of the first structures of the submaxilar gland begins around the 6th week of intra-uterine life, the parotid at the 7th week and sublingual glands about the 9th week.

Salivary glands reach their definitive location at the end of the 3rd month of intra-uterine life, being the parotids the most dislocated from their initial position to oral cavity.

Three stages can be distinguished in the development of salivary glands (Hsu JC and Yamada KM 2010). The first stage is characterized by the emergence of the glandular buttons, constituted by ramifications of primitive small channels around a lumen. The second stage shows a larger differentiation of the gland, with early formation of lobes and ducts canalization. This stage lasts to the 7th month of intra-uterine life, and it determines the formation of the functional units of the gland. The third stage begins in the 8th month of intra-uterine life, leading to the structural maturation of the gland, with differentiation of acinar ductal cells, the ductal system and myoepithelial cells. The development of the glandular tissue is accompanied by the reduction of the initially abundant interstitial connective tissue (Humphrey S and Williamson RT 2001).

Submaxilar glands possess a capsule and are located among fascias, while parotids grow in a niche of subcutaneous tissue, surrounded by connective tissue, therefore not possessing a capsule. Sublingual glands are embedded in a connective tissue that penetrates and divides the glands in their lobes, not having a distinct and homogenous capsule. In the parotid, the first glandular cells appear

above the main trunk of the facial nerve, between their temporofacial and cervicofacial ramifications. The facial nerve divides parotid in superficial and other deep lobules (Espin-Ferra J et al 1991).

6.2. Histology and ultrastructure

The secretory endpieces are composed by serous, mucous and myoepithelial cells organized in acini with secretory ducts. Their secretions are collected in intercalated ducts and released in striated ducts.

6.2. 1. Serous cells

These cells are specialized in synthesis, storage and secretion of proteins. The typical serous cell has a pyramidal shape with the largest base lining a peripheral basal membrane and its vertex pointing to the lumen. The spherical nucleus is located in the basal pole of the cell. The most important aspect of the serous cell is the storage of secretory granules in the apical cytoplasm. These granules have about 1 micron of diameter and, in an electron microscopy, it is possible to see that they have a limiting membrane and a dense and homogeneous content.

The basal portion of the cytoplasm is filled with the endoplasmic reticulum (ER) and a closed system of membranous sacs or cisterns. The ribosomes, constituted by ribonucleic acid (RNA) and proteins are the basic units of proteins synthesis. Under orientation of the RNA messenger, starting from the nucleus, the ribosome translates the codified message, thus uniting the appropriate amino acids, in correct positions, to synthesized specifics proteins. The polypeptides chain in growth is transferred through the membrane of the ER inside the spaces of the cisterns. In the cells that produce great amounts of proteins for secretion, the ER is characteristically well developed and arranged in parallel piles, usually in basal and lateral position to the nucleus, giving to the cytoplasm its characteristic basophilic aspect.

A second system of membranous cisterns - the Golgi apparatus - is located in the apical position or laterally to the nucleus. The Golgi apparatus is constituted by four to six saccules of flat surface, which are lightly curved or in cup form, with the concave portion guided to the secretory surface of the cell. The Golgi apparatus is functionally related with the ER through vesicles that transport the proteins formed in ER. Golgi apparatus is also an important place of addition of carbohydrates residues to the secretory proteins.

With exception of amylase molecules, most of the secreted proteins are glycoproteins. They possess a variable number of oligosaccharide chains, linked to the protein nucleus in serine, threonine and asparagine residues. The carbohydrate moiety of the secretory glycoprotein includes galactose, manose, fucose, glucosamine, galactosamine and sialic acid. Carbohydrate residues addition begins in the ER, but it is completed in the Golgi apparatus. Glycoproteins are stored in secretory granules in the vertex of the cell. Secretion occurs by a process denominated exocytose, which involves the fusion of the granule membrane with the plasmatic membrane in the lumen or in the intercellular duct, following by the opening of the melted portion. This way, the granule membrane is in continuity with the plasmatic membrane and the content of the granule is secreted without cytoplasm loss.

Serous cells contain several other cytoplasm organelles that are also present in other glandular salivary cells. The free ribosomes are in the cellular cytoplasm and are related to the synthesis of non-secretory proteins. The mitochondria are most frequently found among the cisterns of the ER, around Golgi apparatus and along the lateral and basal plasmatic membranes. Mitochondria contain enzymes of the cycle of Krebs (oxidative phosphorylation), constituting the main source of energy necessary for the numerous synthesis processes and cellular transport.

6.2. 2. Mucous cells

They are specialized in the synthesis, storage and secretion of substances. They are different from the ones produced by the serous cells, mainly characterized by two important aspects:

1. they have little or no enzymatic activities, mainly working as a lubricator and tissue protector;

2. carbohydrate proteins ratio is larger and they present large amounts of sialic acid, and occasionally, residues of sulfated sugars.

These differences can be demonstrated by histochemical methods. In histological sections the vertex seems empty, with exception of fine cytoplasm strings forming a trabeculated network. The nucleus and a small cytoplasm border are compressed against the base of the cell. Electron microscopy shows the mucous cell filled with pale secretory droplets, containing a dispersed floccose material.

The nucleus has an oval or flat shape and is located immediately above the basal membrane. The RE is limited to a narrow strip in the cytoplasm along the basal and lateral borders of the cell, and occasionally, among mucous droplets. Mitochondria and other organelles are limited to this strip of basal and lateral cytoplasm. Golgi apparatus is large, consisting of 10 to 12 saccules, located among basal RE and the mucous material. In the concave surface of Golgi apparatus starts the formation of the mucous droplets (Fig. 13). The Golgi apparatus has a very important role in these cells, due to the great amount of carbohydrates that are added to the secreted products.

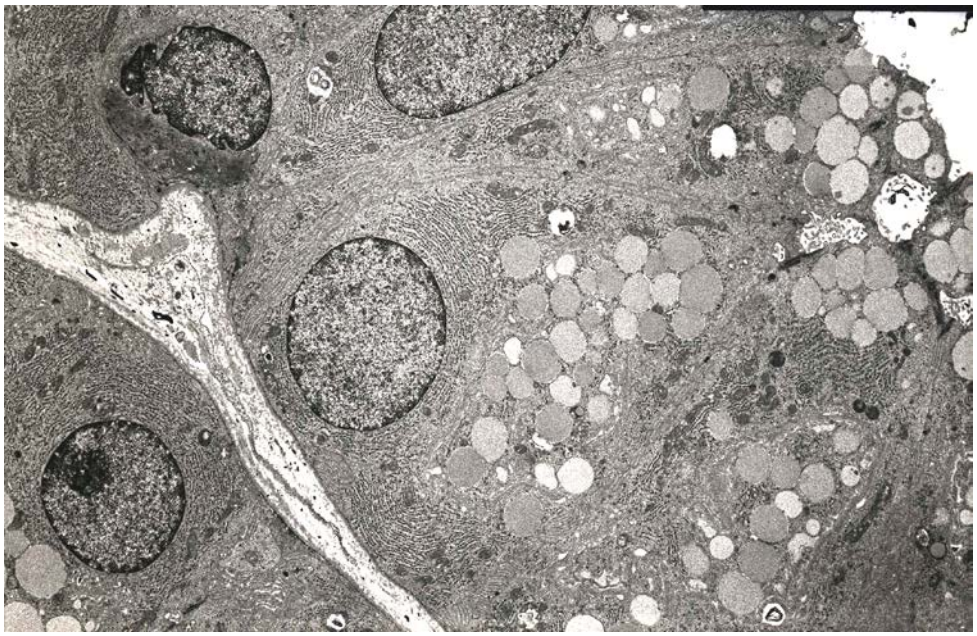


Fig. 13. Salivary mucous cell; the duct is on the right upper corner.
Electron microscopy. x 16.000

The secretion of the mucus droplets begins with a mechanism a little different from the exocytose observed in the serous cells. When a single mucous droplet is released, its membrane merges with the apical plasmatic membrane, resulting in a single membrane separating the droplet of the lumen. This membrane may be lost with the discharge of the mucus, or the droplet can be released with the intact membrane. During the fast discharge of the droplets, the apical cytoplasm cannot close; being the whole mucus mass flowed inside the lumen.

6.2. 3. Myoepithelial cells

These cells are closely related to the secretory cells and intercalated ducts. They are located between the basal part of glandular cells and the basal membranes of the acini (Tucker AS 2007). They are small cells with a flat nucleus and numerous cytoplasm extensions that partially involve the parenchymatous cells. Although they are difficult to identify in routine histological preparations, they are strongly reactive to alkaline phosphatase, being well distinct for their starry or basket shape.

Their cytoplasm contains filaments, mostly actin, tropomyosin and myosin arranged in a similar way as in smooth muscular tissue. Myoepithelial cells have contractile properties, increasing the pressure inside the ducts and facilitating salivary flow rate.

6.2. 4. Cellular disposition in terminal secretory units

The structure of secretory endpieces is different in each gland. In a gland totally constituted by serous units, as the human parotid, serous cells are organized in a way grossly spherical around a central lumen, forming acini. In the apical extremities of the contiguous cells, the lumen is closed to intercellular lateral spaces, with junctional complexes that maintain glandular cells continuous. This fact avoids the entrance or exit of water, electrolytes, molecules and particles of the intercellular space to the exterior of the organism. Intercellular ducts extend among adjacent cells almost to the base, increasing the secretory surface.

In glands entirely formed by mucous units, the disposition of the secretory cells is similar. It is organized in spherical acini or constituted by a single tubular secretory piece located terminally. The central lumen is larger than the serous acini and the intercellular ducts that are not usually present, being occasionally observed in the mucous cells of the human labial glands.

In mixed glands, the proportion of serous and mucous cells can vary from predominantly serous, as in the submandibular human gland, to predominantly mucous, as in the sublingual gland. Serous units and isolated mucous cells can exist together with secretory units composed by the two types of cells. In this last disposition, the mucous cells form a typical tubular portion, which is covered again in the extremity for crescents of several serous cells, forming demilunes at the periphery of the acini. Secretion of the demilunes serous cells reaches the lumen through the intercellular ducts.

6.2. 5. Ducts

The system of ducts of the salivary glands is progressively arranged by the confluence of small ducts in others of larger caliber. Inside of a lobe, the smaller ducts are intercalated ducts. They are fine ramified tubes of variable length, that link the secretory endpieces to the subsequent larger ducts that are the striated ducts. In connective tissue, interlobular ducts continue joining each other, increasing in size, until the main excretory duct is formed.

6.2. 6. Intercalated ducts

They are covered by a single layer of cuboids cells with a relatively empty cytoplasm. In electron microscopy, the cells of the intercalated ducts share several of the characteristics of the serous cells. A small part of the ER is located in the basal cytoplasm and the Golgi apparatus of moderate size is located in the apical area. In cells close to the secretory units, some small secretory granules are observed. In the periphery of the duct, prolongations of myoepithelial cells are observed linked to the ductal cells by desmosome.

6.2. 7. Striated ducts

They are formed by a layer of high columnar epithelial cells with a large spherical nuclei centrally located. The cytoplasm is abundant and eosinophilic, showing prominent striate in the basal cells extremities, perpendicular to the basal surface. In electron microscopy, these cells have the usual morphology of cells that transport ions: the basal cytoplasm of the cells is divided by deep invaginations of the plasmatic membrane, producing numerous pleats, in the shape of sheets that extend to the lateral limits of the cells and interdigiting with pleats similar to adjacent cells. Mitochondria are abundant and large, usually presenting a radial grow; they are located in the portions of the cytoplasm among the membrane invaginations. In the perinuclear cytoplasm we observe some short cisterns of ER and small Golgi apparatus. Several lysosomes, free ribosome and a moderate amount of glycogen are also present. In the larger ducts, the epithelium is pseudo-stratified, with numerous crescents of smaller basal cells, among high columnar cells. The epithelium of the main duct becomes gradually stratified and ends at the non-keratinized stratified squamous epithelium covering the oral mucosa.

6.2. 8. Connective tissue and vascularization

The glandular acini and the excretory ducts of salivary glands are involved by a loose connective tissue that serves for mechanical and metabolic support, where we can observe fibroblasts, bundles of collagen, reticulin fibers, the amorphous interstitial material of this tissue, some fatty cells and, the migrating cells usually observed in other connective tissue of the organism that include macrophages, lymphocytes, plasma cells, mastocytes and occasionally leukocytes. The cells, as well as the collagen fibers and reticulin, are embedded in a fundamental substance, composed of proteoglycans and glycoproteins.

The vascular structures of the glands are also embedded in a connective tissue, which penetrates in the glands along the excretory ducts, ramifying and following them to the individual lobes.

Striated and intralobular ducts are fed by a dense capillary network, while capillary ansa, that involves the intercalated ducts and secretory endpieces, is less numerous. A system of arteriovenous anastomosis has been described around the larger interlobular ducts.

6.2. 9. Nerves

Acinar cells are innervated by both the sympathetic and the parasympathetic branches of the ANS (Emmelin N 1987). Autonomic nerves are adjacent to both acinar and ductal cells (Fig. 14). The afferent pathways for taste go via the facial and glossopharyngeal nerves to a solitary nucleus in the medulla. There is also input from higher centers in response to smell, sight, etc.

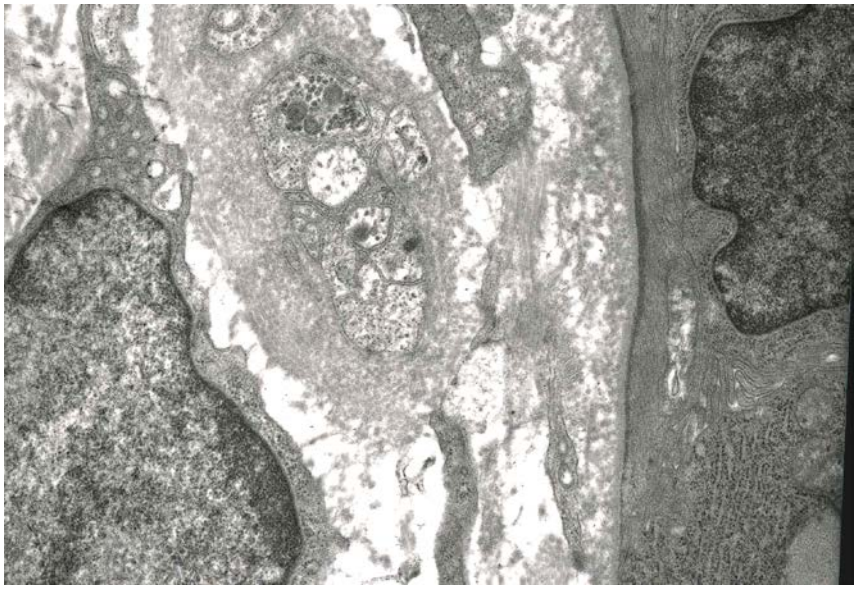


Fig 14: A terminal nerve bundle near a glandular cell.
Electron microscopy, x 8.000

The parasympathetic efferent pathways for the sublingual and submandibular glands are from the facial nerve via the submandibular ganglion; for the parotid gland they are from the glossopharyngeal nerve via the otic ganglion. The sympathetic postganglionic pathways are from the cervical ganglion of the sympathetic chain. Neurotransmitters are the first messengers in the communication pathway between nerves and secretion. Neurotransmitters exert their activity at the cell membrane; they communicate with intracellular second messengers that have direct control of secretory processes. Released in response to secretory stimuli, they bind to specific receptor proteins on the basolateral membrane, causing acute elevation of intracellular Ca. This results in large-scale fluid and electrolyte transport, and exocytosis of stored protein. Norepinephrine from sympathetic neurons binds to both alpha- and beta-adrenergic receptors on the acinar cell. Alpha-receptor activation is linked to elevation of intracellular Ca, while beta-receptor activation causes elevation of intracellular cyclic adenosine monophosphate (cAMP), which is linked to the secretion of salivary proteins that are stored in membrane-bound secretory granules (Ambudkar IS 2011).

6.2. 10. Histological and ultrastructural aspects of labial salivary glands

Labial salivary glands are located in the inferior lip submucosa involved by connective tissue or, less frequently, between muscular fibers. They do not possess a capsule and are organized in lobular structures with secretory endpieces and ducts opening up in the mucosa. They are predominantly mucous salivary glands; cells are wide with a flat nucleus located towards the basal lamina: the cytoplasm is full of mucous granules. Some glands are seromucous, possessing serous cells with a spherical nucleus, a basophilic cytoplasm and numerous secretory granules apically located (Fig. 15). Myoepithelial cells are present in secretory terminal pieces and in small ducts. They possess filaments of actin, which with its contraction helps, as in the others salivary glands, to eliminate the content of the ductal acinar cells whose volume is very low. The ductal system is less developed than in the parotids and submaxilar glands, seeming the intercalated ductal system of the other glands.

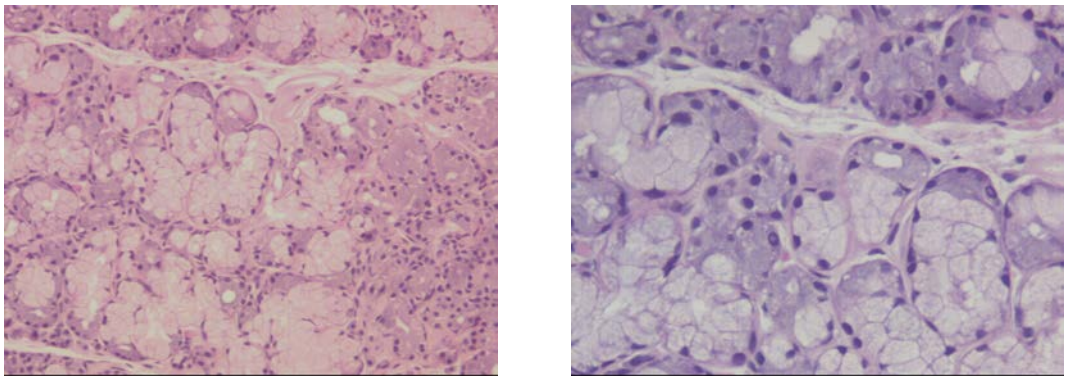


Fig. 15: Labial salivary gland: serous and mucous areas and semi-lunar formations. Left: H. – E., x 160; right: H- Congo Red x 320

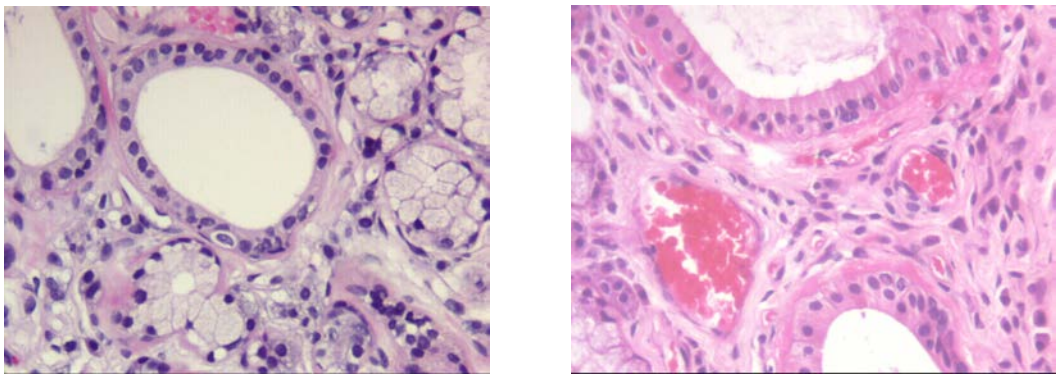


Fig. 16: Intercalated ducts on the left and striated ducts on the right. H.-.E. X320

7. Saliva functions

The acinar cells of the parotid gland have constituted an investigation model in order to study the secretory mechanisms of the exocrine glands (Gorr SU et al 2005).

Salivary glands segregate a primary fluid with a constant concentration of sodium, which is later modified by a process of reabsorption along the ductal system.

Secretion is controlled by the autonomic nervous system through a complex system of cellular signaling responsible for ion secretion. Saliva volumes produced vary depending on the type and intensity of the stimuli, being larger when upon cholinergic stimuli. The neurotransmitters released link to a specific local receptor in the membrane of the acinar cells. In cholinergic stimulation, the system of cellular signaling involves the release of intracellular calcium (Ambudkar IS 2011).

As for the rest, there is a continuous salivary flow without any external influence that is very likely to happen, due to the spontaneous activity of the salivary central nuclei. Larger amounts are segregated above the basic needs, as an answer to varied stimulus such as taste, smell or mastication (Hodson NA and Linden RWA 2006).

7. 1. Salivary ducts functions

Intercalated ducts work mainly as passive drivers between the terminal secretory units and the striated ducts. However, the presence of some cells with apparent secretory granules indicates a possible contribution for the primary secretion. The structure of the striated ducts suggests that they take a more active part in the modification of the primary secretion. Accumulation of granules and vesicles in the cells vertices suggest secretion or reabsorption for pinocytosis (Gorr SU et al 2005).

The cells of salivary ducts have an important role in the saliva characteristics. Composition of saliva will be described later, but the regulatory function of these cells is exemplified in several studies. As an example, analyses of the primary secretion of a lumen of an intercalated duct obtained by micropuncture techniques reveal that it is isotonic or slightly hypertonic comparatively to plasma, with sodium and chlorine concentrations approximately similar to those in the plasma. The potassium concentration is low, compared to sodium, but it is significantly higher than the plasma concentration of potassium.

Analyses of the fluid obtained from the secretory ducts reveal that it is hypotonic, with low concentrations of sodium and chlorine and high potassium concentrations. Besides, the concentration of these electrolytes varies with the intensity of the salivary flow: with the increase of the flow, the sodium and the chlorine also increase, while potassium decreases. The striated ducts actively reabsorb sodium of the primary secretion and secrete potassium; the chlorine tends to follow the electrochemical gradient established for the sodium reabsorption. With increased flow, reabsorption of sodium becomes less efficient, and the secretion is in contact with the epithelium of the duct for a shorter time. Consequently, the saliva concentrations of sodium tend to increase (Almståhl A et al 2003).

8. Saliva composition

Saliva is an aqueous solution of several substances of low and high molecular weight. Electrolytes, proteins and water are the most important products, independently of the places of their production (Schenkels LC et al1995).

Salivary pH, salivary flow and the content in inorganic phosphates show the same rhythm as oral temperature, with a minimum about 06.00 AP and a maximum value in the afternoon, about 06.00 PM. Sodium, iodine and chlorine have opposite variations, while the concentration in potassium does not change in 24 hours. The normal salivary pH locates between 6.0 and 7.0 being therefore lightly acidic. The buffer capacity of saliva is an important factor that influences the salivary pH and the process of dental remineralization, being the total salivary flow and its bicarbonate concentration the most important factors (Fenoll-Palomares C et al 2004).

In non-stimulated parotid secretion, the potassium concentration is larger than the sodium, while after stimulation this relationship is inverted.

Salivary calcium is in the ionized form and is also bound to proteins, in a ratio of 1:1. Its total concentration is larger in the non-stimulated submandibular saliva (2-8 mmol/L) than in the non-stimulated parotid secretion (0.2-2.5 mmol/L).

Chlorine ion concentration in non-stimulated parotid and submandibular glands accompanies the rhythm of sodium concentration of and reaches about 17 to 22 mmol / L. (Almståhl A et al 2003).

The concentration of total proteins in the saliva depends on the methods used in their assessment (Michishige F et al 2006). A lot of salivary proteins that include digestive enzymes as the alpha-amylase, and also glycoproteins (mainly responsible for the saliva viscosity) reach saliva by exocytose (Zussman E et al 2007).

Other constituents as albumin and immunoglobulins reach saliva for probable diffusion from serum, depending on its concentration gradient.

The daily volume of saliva production is 1-1, 5 litter (Dawes C 2008). There is also great variability regarding the contribution of the different salivary glands for the total daily volume. The highest volumes are attributed to the submaxilar glands as they are responsible for the salivary secretion at rest. The volume of the

parotid secretion can exceed the volume of the submaxilar glands, as a response to stimuli such as food.

The contribution of the minor salivary glands is difficult to determine, varying among 7-8% of the total of the saliva (Ogami K et al 2004). Decrease in saliva production has deleterious effects in the oral cavity and in health in general (Dodds MW et al 2005). In several studies with healthy individuals medium values of salivary flow of 0.3ml /min were obtained. Lower values the 0.1ml / min are defined as hyposalivation (Nederfors T 2000). Xerostomia is subjectively defined as the sensation of dry mouth, which may be or not accompanied of the decrease of the glandular function, or in reduction in the secretion of the non-stimulated saliva (Dawes C 2004).

The main methods for saliva collection are spontaneous drainage, expectoration, and collection with a vacuum capsule, with a hypodermic syringe or with cotton rolls. It is difficult to predict normal values for salivary secretion at rest and stimulation, due to the different conditions of saliva collection (Navazesh M and Kumar SK 2008).

The reduction of the salivary flow can have an impact in patients, giving them a few symptoms, such as speech and swallowing difficulties, being therefore its re-establishment of major importance in the maintenance of the oral health (Navazesh M 2003).The individual variation can be as high as 50% in a 24 hours period associated with circadian rhythms and the climate (Kariyawasam AP and Dawes C 2005) and are age and sex independent (Ghezzi EM et al 2000). Other factors can influence salivary secretion, such as food ingestion (Engelen L et al 2007), physical activity (Chicharro JI et al 1999) and medication (Moore PA and Guggenheimer J 2008).

There are multiple causes for the decrease of the salivary flow as diseases of the oral cavity, (Mathews SA et al 2008), systemic diseases (von Bültzungslöwen et al 2007), chemotherapy (Mazzeo MA et al 2009), radiotherapy (Shiboski CH et al 2007) and psychological factors (van Stegeren AH et al 2008).

8.1. Functional significance of salivary constituents

Salivary constituents carry out several functions. The specific function of many salivary components is unknown, as they reach saliva by passive diffusion from serum.

Although the main function of saliva is digestive, some substances that are excreted in saliva provide defense mechanisms of the mouth. In the absence of

saliva, there is a progressive deterioration of teeth and of the oral mucosa. Taste alterations and speech becomes more and more difficult.

The digestive function of saliva begins with the preparation of the alimentary bolus that precedes its digestion in the gastrointestinal tract. The alimentary bolus is lubricated to reduce the friction during swallowing. This saliva lubricant function is mainly achieved by its content in glycoproteins. They are the base of the mucous matrix in any part of the organism and are present in varying concentrations in saliva of labial, sublingual and submaxilar salivary glands. This explains the variation of the viscosity of the salivary secretion at rest, favoring the soft intra-oral movements, as for instance, during speech. The efficiency of saliva as a lubricant depends on its viscosity (Stokes JR and Davies GA 2007).

It has been found that salivary viscosity is greatly influenced by pH and calcium. Salivary viscosity is also suggested to contribute to denture retention. This dynamic process dependent on the control of the flow of interposed fluid between the prosthesis and the oral mucosa (Christersson CE et al 2000). Alterations in salivary composition are reflected in its viscosity and can cause oral complaints (Chimenos-Kustner E and Marques-Soares M 2002).

Enzymatic digestion of food begins in saliva. The main digestive salivary enzyme is alpha-amylase, which is produced in the parotid gland and it is released in the saliva by zymogene granules (Noble RE 2000). It consists of two families of isoenzymes, one of which is glycosylated and the other contains no carbohydrate (Fisher SZ et al 2006). The concentration of amylase increases with the salivary flow rate and it is generally considered to be a reliable marker of serous cell function (Almståhl AM et al 2001). In addition to its well-known function as a digestive enzyme, amylase has been reported to act as an antimicrobial enzyme. Amylase interacts specifically with certain oral bacteria and plays a role in modulating the adhesion of those species to teeth (Ragunath C et al 2008). Amylase activity also exist in tears, nasal and bronchial secretions, milk, serum, urine and in secretions of the urogenital tract (Tenovuo J 1989). It has been suggested that there is a good correlation between amylase and pain scale in patients with chronic pain (Shirasaki S et al 2007).

Albumin is the most abundant serum protein, constituting more than 50% of all of the plasmatic proteins. Its molecular mass is 65 kDa and the normal serum reference limits are 40-52 mg/L. Albumin is synthesized exclusively in the liver at a rate of 100-200 mg/kg/day. Factors that regulate albumin synthesis are nutrition, hormonal balance and osmotic pressure. Nephrotic syndrome is the

best known example of a systemic disorder with characteristic proteinuria and subsequent hypoalbuminemia - which leads to edema (Ducloux D 2011)

The albumin in the oral cavity is not filtrated, diffusing passively into mucous secretions (Schenkels et al 1995). Salivary albumin is increased in immunosuppressed patients (Lin A et al 2003), patients undergoing radiotherapy (Almståhl A et al 2001) and diabetic patients (Dodds MW 2000). Patients with graft-versus-host-disease suffer from oral dryness, increased oral infections and mucosal pathologies. Salivary albumin is reduced in those patients (Nagler RM and Nagler A 2003).

Salivary albumin can be used as a marker of the integrity of the oral mucous membrane (I Aviv et al 2009). In periodontitis albumin salivary values are very high (Henskens YM et al 1993). There are a significant correlation between the gingival index and salivary albumin in diabetic patients (Khader YS et al 2006). A relationship between the concentrations of serum albumin and the radicular decays was found (Bardow A et al 2005). Albumin concentrations are higher than in the control groups, in both parotid and whole saliva in primary Sjögren syndrome patients. However, the output/min of albumin was lower is SS patients (Cuida M et al 1997).

Salivary urea is metabolized in ammonia and carbon oxide in the oral cavity. Results from a modeling study suggest that the salivary urea concentration is sufficient to raise the plaque pH minimum after a sucrose rinse by at least one-half pH unit (Dibdin G Dawes C 1998). A relation between the development of dental caries and salivary urea levels in healthy human and some diseases has been suggested (Nascimento MM et al 2009).

Salivary mucins have a multifactorial role in the oral cavity as they lubricate oral surfaces, provide a protective barrier between underlying hard and soft tissues and the external environment, and in mastication, speech and swallowing as well as in the non-immune host defense system in the oral cavity (Tabak LA 1995). They are constituted by two glycoproteins, one of high-molecular mucin (MG1) and another of low-molecular weight (MG2). From a biochemical standpoint, mucins represent approximately 15%-20% protein and up to 80% carbohydrate - present largely in the form of O-linked glycans (Gendler SJ and Spicer AP 1995). Serine and threonine are the most abundant amino acids that work as the attachment sites for these carbohydrate chains (Offner GD and Troxler RF 2000). To date, 11 distinct human mucin genes have been isolated and have been numbered MUC1-MUC4, MUC5AC, MUC5B, MUC6-8, and MUC11-

MUC12 in the order of their discovery. Mucins could be organized into three distinct classes: the large gel-forming mucins MUC2, MUC5AC, MUC5B, andMUC6; the large membrane-associated mucins MUC1, MUC3, MUC4, and MUC12; and the small soluble mucins represented by MTJC7 (Gendler SJ and Spicer AP 1995).

MG1 binds to hydroxyapatite and it is thought to be a constituent of the acquired enamel pellicle covering the surfaces of hard tissues in the mouth (Al-Hashimi I and Levine MJ 1989). In addition, MG1 is an important component of the innate host defense system in the oral cavity, since it has been shown to bind and facilitate clearance of a variety of salivary microbes, including the periodontal pathogen - Porphyromonas gingivalis - (Amano A et al 1994), and the opportunistic yeast Candida albicans (Edgerton M et al 1993), providing an inhibitory mechanism of Human Immunodeficiency Virus type 1 (Habte HH et al 2006).

8.2. Salivary antimicrobial proteins

Saliva contains several substances that play an important role in resistance to infections. They protect the oral cavity, this one being an entrance door to many microorganisms present in foods or in the air, and also in infected material.

Such substances include histatin, protein rich-proline, immunoglobulins, a group of heterogeneous glycoproteins and others.

Salivary histatins (Hts) are a family of histidine-rich cationic proteins produced by human salivary gland acinar cells that are key components of the innate defense system in the oral cavity. Hst 5 has high potency against Candida albicans at physiological concentrations (10 to 30 μM) (Jang WS et al 2010), including azole- or amphotericin-resistant strains of Candida (Tsai H and Bobek LA 1998).They represent a major component of the innate host non-immune defense system. All human major salivary glands are involved in the secretion of histatins into saliva. Human von Ebner's glands located in the posterior region of the tongue represent a significant source of histatins, supporting the hypothesis of their important role in preventing microbial assaults on the tissues in the posterior region of the tongue and in the circumvallated papillae (Piludu M et al 2006).

Human salivary proline-rich proteins (PRPs) constitute a significant fraction of the total salivary proteins and have important biological activities. They are present in the initial phase of the formation of the acquired bacterial film and

also in mature pellicles (Siqueira WL and Oppenheim FG 2009). Parotid salivary basic proline-rich proteins inhibit HIV-I infectivity (Robinovitch MR et al 2001). They are still promoters of the adhesiveness of many bacteria to hydroxyapatite (Drobni M et al 2006). They also have lubricator roles in saliva and interact with other biological compounds forming complexes with them, thus preventing their absorption from the intestinal tract (Petti S and Scully C 2009).

Lysozyme represents the main enzyme of the nonspecific salivary immune defense and is secreted mainly by submandibular and sublingual glands (Noble RE 2000). It is also known that lysozyme contributes to mucosal protection and modulates Candida species in the oral cavity (Anil S et al 2001).

Peroxidases and myeloperoxidases catalyze a reaction involved in the inhibition of bacterial grow and metabolism, as well as in the prevention of hydrogen peroxide accumulation (Ihalin R et al 2006).

Lactoferrin binds iron thus making it unavailable for microbial use: iron exerts a direct bactericidal effect on some bacteria including species of Streptococcus mutants (Loimaranta V et al 1998).

The immunoglobulin G (IgG) and M (IgM) are present in small amounts in saliva and are a partial mixture of serum immunoglobulins. The main specialized immunoglobulin isotype of the secretory immune system is secretory immunoglobulin A (s-IgA) - the major antibody in saliva. Two IgA molecules linked by a J-chain are synthesized by plasma cells associated with salivary glands. This dimeric IgA then binds by its J-chain to a receptor for polymeric immunoglobulin (pIgR) on the cell membrane of the salivary gland epithelium (Corthésy B and Spertini F 1999). This complex is transported across the epithelial cell in an endocytic vacuole and enters the salivary duct by its luminal surface where cleavage of the pIgR receptor releases secretory IgA into saliva, with a portion of the pIgR receptor, the secretory piece, still attached. The s-IgA inhibits the adherence of the microorganisms to the oral epithelium and the teeth and it is early present in high amounts in the saliva of the new born (Nogueira RD et al 2005). At least 95% of IgA present in the saliva is produced in the parotid glands. There are high levels of s-IgA in insulin- dependent diabetic patients (Ben-Aryeh H et al 1993), in pregnancy (Bratthall D and Widerström L 1985), and in patients infected by the human immunodeficiency virus (Mellanen L et al 2001). Minor salivary glands, particularly the labial glands, play an important role in the immune system of the oral cavity by producing higher IgA concentrations (Ferguson DB 1999).

Salivary agglutinins are glycoproteins which have the capacity to interact with unattached bacteria, resulting in clumping of bacteria into large aggregates, which are more easily flushed away by saliva and swallowed (Ahn Sug-Joon et al 2008). They can mediate the adherence of several bacterial species to the teeth (Stenudd C et al 2001). Salivary agglutinin inhibits HIV type 1 infectivity through interaction with viral glycoprotein 120 (Wu Z et al 2003).

9. Saliva as a diagnostic fluid

Whole saliva can be collected non-invasively, requiring no special equipment, which is potentially valuable for children and older adults, since collection of the fluid is associated with a fewer compliance problems when compared with collection of blood. Advances in the use of saliva as a diagnostic fluid has been affected by current technological developments. For example, it is possible to compare a wide range of molecular components in saliva with the same components present in serum and to study microbe, chemicals and immunological markers (Slavkin HC 1998).

The major advantages for using saliva in diagnosis rather than blood have been described in depth earlier (Ferguson DB 1987; Malamud D 1992; Mandel ID 1993; Slavkin HC 1998). The methodology to select the type of total saliva versus individual glandular saliva has also been described (Mandel ID 1980; Sreebny LM and Zhu WX 1996). The specific collection methodology to be used (Navazesh N 1993) and the physiological factors affecting salivary collection have also been reviewed (Dawes C 1993).

There are multiple normal and pathological situations for the use of saliva as a diagnostic fluid:

a) Sjögren syndrome is a chronic, autoimmune disorder characterized by salivary and lachrymal gland dysfunction, serological abnormalities, and multiple-organ changes. Sreebny and Zhu 1996 proposed a panel of salivary determinants that could be clinically used for the diagnosis of Sjögren syndrome that include flow rate, pH, buffer capacity, lactobacillus and yeast concentration. Other investigators suggested that interleukin 2 (IL-2) and interleukin 6 (IL -6) are significantly elevated among individuals suffering from Sjögren syndrome (Streckfus C et al 2001);

b) Cystic fibrosis is a genetically transmitted disease of children and young adults which involves a generalized exocrinopathy. The elevated calcium and phosphate levels in the saliva of children diagnosed with CF may explain the fact that these children demonstrate a higher occurrence of calculus when compared with healthy controls (Wotman S et al 1973).The alterations of salivary lipids in CF patients may account, in part, for the altered physicochemical properties of saliva in these patients (Slomiany B et al 1982). The excretion of an unusual, less effective form of salivary epidermal growth factor was reported (Aubert B et al 1990);

c) Coeliac disease is a congenital disorder of the small intestine that involves malabsorption of gluten. Gliadin is a major component of gluten. Serum IgA antigliadin antibodies (AGA) are increased in patients with coeliac disease. Measurement of salivary IgA-AGA has been reported to be a sensitive and specific method for the screening of coeliac disease and for monitoring compliance with the required gluten-free diet (Hakeem et al 1992).

d) Stress: salivary α-amylase has a more direct and simple method to study the catecholamine activity than the changes in heart rate when evaluating patients under a variety of stressful conditions. Salivary α-amylase appears to be higher under conditions of extreme examination stress (Nater U et al 2009);

e) Infections: Helicobacter pylori infection is associated with peptic ulcer disease and chronic gastritis. Saliva samples were tested for the presence of H. pylori DNA by polymerase chain-reaction (PCR) assay, being sensitivity levels of 84% reported. The results also indicated that H. pylori existed in higher prevalence in saliva than in feces. The oral-oral route may be an important means of transmission of this infection in developed countries. A variety of others infections has been monitored by the detection of specific antibodies in saliva: Sigella, Pigeon breeder's disease, antibody for Lyme disease, neurocysticercosis, the diagnosis of viral hepatitis A (HAV) and hepatitis B (HBV), determination of immunization and detect infection with measles, mumps and rubella, as a marker for rotavirus infection and epidemiological surveillance of HIV.

f) medication: in patients receiving lithium therapy, carbamazepine, cyclosporine, theophylline, anti-cancer drugs, alcohol levels, amphetamines, barbiturate, benzodiazepines, cocaine, phencyclidine and opioids and cotinine levels;

g) endocrine function: cortisol levels were found to be useful in identifying patients with Cushing syndrome and Addison disease, in patients with primary aldosteronism in hyperandrogenic women, in behavioral studies of aggression, depression, abuse and violent and antisocial behavior, for the assessment of fetoplacental function, for the prediction of ovulation, as markers of fetal grow retardation, as a predictor of pre-term delivery and in the glucose tolerance test;

h) tumor markers: Antibodies against p53, tumor markers c-erb-B-2 (erb) and cancer antigen 15-3 (CA 15-3) and CA 125 may be also studied by this methods. This subject will be developed later;

i) others applications: saliva can be used for monitoring the effects of multiple medications in the wellbeing of the individuals as diuretics, antihypertensive, antihistamines, antidepressants, anticholinergic, antineoplastic opiates, amphetamines, barbiturates, hallucinogens, cannabis and alcohol have been associated with a reduction on salivary flow. Reduced salivary flow may lead to oral problems such as progressive dental caries, fungal infections, oral pain and dysphagia. Increased levels of albumin in wile saliva were detected in patients who received chemotherapy as treatment for cancer, subsequently developing stomatitis. Monitoring of salivary albumin can assist in the identification of stomatitis at a pre-clinical stage and enable the chemotherapy dosage to be adjusted, or treatment for the stomatitis to be initiated at an early stage.

10. Salivary genomics, transcriptomics and proteomics

In recent years, genomics, transcriptomics and proteomics of saliva became popular, since they represent a non-invasive, safe and inexpensive source of complex genetic information.

Salivary DNA represents the genetic information (genome) of the hosting human body of the oral microbes present in the mouth and the infecting DNA-viruses. Salivary mRNA provides information on the transcription rates of host

genes (the human oral transcriptome) and those of the oral microbiota. Other salivary RNA may indicate RNA-virus infection. Salivary protein (proteome) represents genetic information and provides help to understand the translational regulation of the host body and the oral microbiota (Park NJ et al 2006).

The value of total DNA content in human saliva was found to range between 1.8-128.4 µg/ml, with a mean value of 21.6 4 µg/ml (Hansen TVO et al 2007). Although it has not been yet investigated in detail, it is likely that desquamated oral mucosal cells represent the main human DNA source of saliva.

Some authors hypothesize the absence of human mRNA in cell free saliva. Hence, high caution should be taken in the interpretation of saliva-based mRNA expression studies without the technical exclusion of possible DNA contamination (Kumar SV et al 2006, Park J et al 2007).There are roughly 200 salivary mRNA found in all individuals termed the “normal salivary transcriptome core” (Li Y et al 2004; Hu S et al 2007).

The total concentration of proteins in whole saliva ranges between 0.5 to 3.0 mg/ml. This proteome consists of roughly 1.000 distinct protein sequences from which around 300 sequences are of human origin. However, only a low correlation of the change of the amount of a certain protein and the alteration of its mRNA level was found under a particular pathological condition, such as the Sjögren syndrome (Alevizos I et al 2011) and dental caries (Vitorino R et al 2005).

The microbiota of the oral cavity consists of more than 600 microbial species including predominantly bacteria, but also fungi and protozoa (Kang J-G et al 2006). However, in individual hosts only a subset of all species can be found indicating a high degree of interpersonal variability (Li Y et al 2005).

Despite the important antimicrobial activity of saliva, the complexity of salivary microbiota was similar, including 33 to 53 different bacterial species and phylotypes (Li Y et al 2005), and 1 to 3 yeast Candida species (de Jong MH and Van der Hoeven JS 1987). Increased expression of molecular chaperones strongly increases the acid tolerance of salivary bacteria as Streptococcus mutans on pH 5. Such a low pH occurs regularly in the deeper area of tooth surfaces with attached bacterial biofilm forming the dental plaque indicating that many bacteria present in such biofilm may survive in rather low pH. Such bacteria may also survive passing through the stomach (Lemos JA et al 2001).

11. Salivary disease biomarkers

11.1. Tumor pathology

There are some tumor specific DNA markers in serum and other body fluids that may be used for the diagnosis of tumors, including oral tumors, based on the assumption that the initiation and progression of malignant tumors is driven by the accumulation of specific genetic alterations (Li Y et al 2005).

For patient risk assessment, the detection of DNA in saliva of tumor-inducing viruses, such as human immunodeficiency virus, human herpesvirus-8 and the detection of human papilloma virus (HPV) DNA in saliva.

Profiling of salivary mRNA indicated four major biomarkers of oral cancer including interleukin-1-β (IL 1β), interleukin-8 (IL 8), ornithine decarboxylase antizyme (OAZ 1) and spermidine/spermine N1-acetyltranferase (SAT).

Regression tree analysis of this four elevated biomarkers was able to distinguish patients with T1 and T2 oral cancer from control subjects with very high sensitivity 91%) and specificity (91%) (Li Y et al 2004). Detection of tumorigenic virus RNA, such as HIV-1 may also be useful for evaluation of risk of malignancies (Freel SA et al 2001).

Salivary proteome can also be used for tumor detection. Increased salivary level of defensin-1 (Misukawa N et al 1998) and cancer antigen CA15-3 were found (Agha-Hosseini et al 2009). Tumor marker protein, such as c-erbB-2 and antibodies against the tumor suppressor protein p53 (Tavassoli M et al 1998) seem to be promising markers of both oral and other malignancies.

11.2. Sjögren syndrome

Sjögren syndrome has been widely studied. There are 16 down-regulated proteins and 25 up-regulated distinct amino-acid sequences in whole saliva proteome, from which 10 up-regulated and 6 down-regulated proteins seemed to be statistically significant biomarkers (Hu S et al 2007).

11.3. Oral candidiasis

Alterations of salivary proteome related to proteins showing antifungal properties such as immunoglobulin, calprotectin, hystatin-5, mucins, peroxidases, basic proline-rich proteins and molecular chaperone Hsp70 may also have importance in cases of recurrent oral candidiasis.

11.4. Caries and periodontal diseases

There are some reasonable specific changes of the salivary proteome, which may be used for recognizing caries-risk patients. These include decreased level of proline-rich proteins (PRP1 and PRP 3), histatin I and statherin (Vitorino R et al 2005).

The presence of certain periodontopathogen bacterial species in saliva reflect their presence in dental plaque and periodontal pockets (Boutaga K et al 2007), indicating that saliva may be a good tool for detecting bacterial risk factors by salivary DNA analysis of oral microbiota.

The level of alpha-2-macroglobulin, alpha-1-antitripsin, elastase and albumin in saliva may be good indicators of gingivitis and/or periodontitis. The level of salivary defense protein such as immunoglobulin, molecular chaperone Hsp70, cystatin S, salivary amylase, calprotectin, histatins, lysozyme, lactoferrin, defensins, peroxidases, proline-rich-proteins and mucins may also have prognostic value related to the possible passing of gingivitis to periodontitis, where the latter is more a severe inflammation with irreversible destruction (Fábián TK et al 2007).

11.5. Familial amyloidotic polyneuropathy

The adipokine ZAG detected in FAP saliva is a potential biomarker to be explored, as ZAG can be related to the cachexia profile that FAP patients present in the later stages of the disease (Macedo B et al 2007).

II. OBJECTIVES

OBJECTIVES

The main objective of this thesis is to describe and demonstrate the presence of oral manifestations in FAP.

Studies in Swedish patients have demonstrated the presence of xerostomia as a major oral manifestation in FAP. Xerostomia was also one of the symptoms described by patients with FAP in a study of Portuguese patients with digestive manifestations.

In this thesis we aim to study the main oral manifestations in FAP, along with the demonstration of the presence of amyloid in the labial salivary glands, as one of the factors that may contribute to patients having dry mouth.

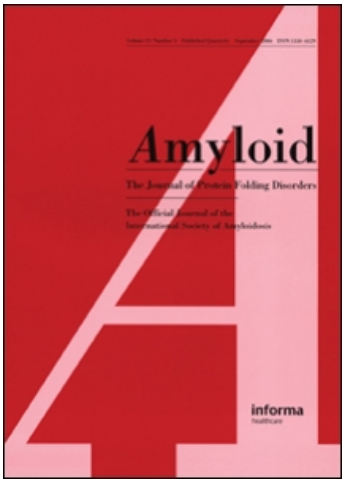
We set out to demonstrate that the biopsy of labial salivary glands obtained the same results as the nerve biopsy, which has previously been used at the Hospital of Santo Antonio in Porto, being the method of choice for the demonstration of amyloid. We sought to establish a relationship between amyloid deposits and stages of disease, in order to identify some biomarkers that may be involved in the gland, along with its relationship with the various stages of disease and the results obtained with transplantation and medical treatment of FAP.

Specific objectives

- Be aware of the incidence of amyloid deposition in FAP;
- Study the distribution of amyloid deposits in labial salivary glands and characterize them;
- Clarify whether there is any relationship between the histological pattern and clinical expression;
- Carry out a qualitative and quantitative study of the components of saliva;
- Demonstrate the existence of hyposalivation and its relation to the stages of the disease;
- Identify biomarkers of glandular disease at an earlier stage of the disease.

III. USEFULNESS OF LABIAL SALIVARY GLAND BIOPSY IN FAMILIAL AMYLOID POLYNEUROPATHY PORTUGUESE TYPE

Do Amaral, Barbas, Coelho, T., Sousa, and Guimarães A. (2009). Usefulness of labial salivary gland biopsy in familial amyloidotic polyneuropathy Portuguese type". Amyloid, 16:4, 232- 238.



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Usefulness of labial salivary gland biopsy in familial amyloid polyneuropathy Portuguese type

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Usefulness of labial salivary gland biopsy in familial amyloid polyneuropathy Portuguese type

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Keywords: *TransthyretinVal30Met, amyloidosis, labial salivary gland biopsy, FAP patients*

Abbreviations: *FAP = familial amyloid polyneuropathy; LSG = labial salivary gland; TTR = transthyretin*

Abstract

Background. The diagnosis of amyloidosis of all types is definitively made by demonstration of Congo red binding material in the affected tissues. Nerve biopsy was classically used to diagnose amyloid polyneuropathy but less invasive alternative types of biopsies have been proposed including labial salivary gland (LSG) biopsy, a minimally invasive procedure.

Method. LSG biopsies were done in 87 subjects with molecular diagnosis of TTRVal30Met mutation. The group includes 76 patients in different stages of familial amyloid polyneuropathy and 11 asymptomatic carriers. They were all submitted to a stomatological and a neurological observation to evaluate oral health problems and to determine the neurological stage of the disease. No major oral health problems were found. Mean age of onset of the symptomatic disease was 32.8 years ($\pm$9.69 SD).

Conclusions. No significant side effects occurred after the surgical procedure, and adequate material for pathological analysis was always obtained. Amyloid deposition was found in 91% of the patients. Patients with negative biopsies ($N = 7$) were all in the earlier stage of the disease. Two asymptomatic carriers had biopsies with amyloid deposition. We conclude that LSG biopsy is a useful, sensitive and minimal invasive method to detect amyloid deposition.

Introduction

Familial amyloid polyneuropathy (FAP) is an inherited form of amyloidosis first described by Corino de Andrade in families from northern Portugal [1]. The main clinical feature is a highly disabling ascending autonomic, sensory and motor neuropathy that is fatal, on average, 11 years after onset [2]. Autonomic neuropathy is responsible for severe gastrointestinal, cardiocirculatory and genitourinary problems. Renal, ocular and myocardial involvement may also occur in some patients [3–6].

The disease is due to the deposition of a variant protein transthyretin (TTR) as amyloid substance. More than 100 amyloidogenic mutations have been identified [7], but TTRVal30Met is by far the most common and the one responsible for the large foci identified in Portugal, Sweden, Majorca and Japan [8–11].

Nowadays, the diagnosis of FAP TTR Val30Met is based on molecular analysis of the TTR mutation. However, as the mutation is present at birth and clinical symptoms are non-specific, the demonstration of amyloid deposition remains the gold standard for the diagnosis of the onset of the disease, particularly when invasive procedures such as liver transplantation are proposed as a treatment.

Nerve biopsy has been used in the past as a common diagnostic procedure but less invasive biopsies such as abdominal fat, skin or rectal mucosa are now preferred. Labial salivary gland (LSG) biopsy is a simple procedure, minimally invasive, commonly used to diagnose Sjögren's syndrome.

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It has also been described as an easy tool for the demonstration of amyloidosis [12,13].

To understand the influence of the disease in the development of oral pathology and the usefulness of LSG biopsy to demonstrate amyloid deposition, we systematically studied a group of 87 carriers of the mutation. Of these, 76 were patients in different stages of the disease, the majority of which were evaluated in the context of their preparation for liver transplantation. A group of 11 asymptomatic carriers were also included to characterise pre-clinical stage of the disease.

We present in this article the pathological data from LSG biopsies and we correlate them with some clinical aspects, emphasising the relation between that data and the oral problems and the clinical stage of the disease.

Patients and methods

Subjects

Our study involved 76 patients (41 men, 35 women) and 11 asymptomatic subjects (6 men, 5 women). All subjects had the TTRVal30Met mutation determined by DNA analysis and a neurological observation to establish the medical history and define the clinical stage. The staging system used was as follows: P0 – asymptomatic with a normal neurological examination; P1 – sensory disturbances in the lower extremities and mild autonomic disturbances; P2 – sensory and mild motor disturbances in the lower extremities, mild sensory disturbances in the hands and moderate autonomic disturbances; P3 – difficulty in walking (need for orthothesis or support), moderate sensory and motor disturbances in the hands and moderate autonomic disturbances; P4 – patients in a wheelchair or bed ridden, with severe autonomic neuropathy [14].

The Ethical Committee of Hospital de Santo António approved this study and all subjects gave written informed consent before the LSG biopsy.

Labial salivary gland biopsy

LSG biopsy was performed using local anaesthesia (3% Lidocaine). The lip was everted, and a 0.5–10 mm longitudinal incision was made in the labial mucosa in front of the mandibular canine tooth and sutured with a reabsorbable monofilament. Two to four LSG were removed.

Histopathological study

One gland was fixed in a 10% formaldehyde solution and studied by optical microscopy; another gland was fixed by immersion in a 2.5% buffered glutaraldehyde, post-fixed in 1% osmium tetroxide, embedded in Epon and prepared for semi-thin sections and electron microscopy examination. Whenever enough material was available, one or two other glands were frozen and stored at $-80^{\circ}C$ to perform immunohistochemistry.

The histological specimens included in paraffin were stained with haematoxylin and eosin to evaluate the gland generally and with Congo red to verify the presence and the distribution of amyloid deposition. The specimen stained with Congo red was always observed in polarised light to confirm the characteristic apple green birefringence.

The global aspect of the LSG and the amount and distribution of the amyloid deposits was graded as follows: G0 – normal morphology without amyloid deposits; G1 – normal morphology of the gland and very discrete amyloid deposits in the interstitial connective tissue stroma or surrounding a few glandular acini; G2 – the global aspect of the gland is preserved with some discrete interstitial fibrosis and more evident amyloid deposits surrounding several glandular acini; G3 – the global aspect of the gland is altered with evident loss of the acinar tissue, pronounced interstitial fibrosis and very abundant amyloid deposits around all glandular tissue (Table I).

Whenever the first slides were negative for amyloid further cuts in deeper portions of the specimen were preformed to confirm the first result.

Immunohistochemistry

Five-micrometer thick sections were deparaffinated in Histoclear® (National Diagnostics, Atlanta, GA) and dehydrated in a descendent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/100% methanol, and sections were blocked with 4% foetal bovine serum and 1% bovine serum albumin in PBS. Primary antibodies used were as follows: rabbit polyclonal anti-Fas (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100), rabbit polyclonal anti-3-nitrotyrosine Chemicon International, Temecula, CA, 1:500), goat

Table I. Morphological classification of labial salivary gland histology.

	Morphological classification			
Salivary gland findings	G0	G1	G2	G3
Normal glandular structure	+	+	−	−
Amyloid				
Connective tissue	−	−/+	+	++
Glandular acini and ducts	−	−/+	++	+++
Loss of acini and ducts	−	−	+	++
Fibrosis	−	−	+	++

polyclonal anti-BiP (Santa Cruz Biotechnology, Santa Cruz, CA, 1:50) and finally rabbit polyclonal anti-TTR (Dako, Carpinteria, CA, 1:1000), which were diluted in blocking solution and incubated overnight at 4°C. Antigen visualisation was performed with the biotin-extravidin-peroxidase kit (Sigma-Aldrich, St. Louis, MO), using 3-amino-9-ethyl carbazole (Sigma-Aldrich, St. Louis, MO), or diaminobenzidine as substrates. On parallel control sections, the primary antibody was replaced by blocking buffer and by non-immune immunoglobulins corresponding to the species used for primary antibodies, namely rabbit an goat; staining was absent under this conditions.

Variables studied and statistical analysis

We analysed gender, amyloid distribution and disease stage as categorical variables and age-at-onset, age-at-biopsy and duration of disease as continuous variables.

Association between gender of patients and degree of amyloid deposition was assessed using a chi-square test. Two-sample Student's *t*-tests were used to compare mean age-at-onset and mean age-at-biopsy between genders. A 5% significance level was used in all analyses. Statistical analyses were performed using SPSS version 15.0 for Windows (SPSS, Chicago, IL).

Results

Clinical data

The mean age of the patients ± SD (41 males and 35 females) from whom the biopsy specimen was obtained was 37.1 ± 10.4 years (range: 24–73 years). Male patients had a mean age of 37.6 ± 10.2 years (range: 27–68) and female patients had a lower mean age, 36.6 ± 10.7 years (range: 24–73), a difference that was not statistically significant ($p > 0.05$ for the two-sample *t*-test).

The mean age of the asymptomatic subjects ± SD was 32.4 ± 10.9 years (range: 23–61 years). Mean age of male subjects was 35.2 ± 12.9 years (range: 26–61), and mean age of female asymptomatic carriers was 29.0 ± 8.0 years (range: 23–43).

Mean age-at-onset was 32.7 ± 9.9 years for men (range: 23–66) and 32.2 ± 8.9 years for women (range: 21–64). Among Portuguese patients with FAP, women are known to have a significant later onset than men [15], but in this group of patients we found no such difference ($p > 0.80$ for the *t*-test). This is probably due to the predominance of young pre-transplant patients and to the small size of the sample.

The mean duration of symptomatic disease was 4.1 ± 2.6 years (range: 1–14). Median was 3 years, and mode was 2 years.

Neurological evaluation

By definition, all 11 asymptomatic carriers were classified as P0; 45 patients (23 males and 22 females) had only mild sensory and autonomic neuropathy and were classified as P1; 17 patients (7 males and 10 females) had moderate sensory and autonomic neuropathy with mild motor problems and were classified as stage 2; 14 patients (11 males and 3 females) were in stage 3. No stage 4 patients were studied.

When we compared the years of disease evolution with disease stage, we found a large variability in the rate of disease progression: patients in stage 1 vary from 1 to 4 years of symptomatic disease; patients in stage 2 vary from 2 to 14 years of complaints and patients in stage 3 vary from 4 to 10 years. Because of this variability, we considered that disease progression was better expressed by staging, rather than by years of evolution, and we chose to analyse only that variable.

Oral findings

There were no complaints of xerostomia or difficulties in eating, swallowing, tasting or speaking. We have not seen any patient with candidiasis or atypical dental caries (in cervical, incisal surfaces or cusp tip locations). Some patients had tooth loss, and marginal periodontitis caused by dental plaque. There was no clinical evidence of amyloid deposition in the oral cavity, namely the presence of macroglossia.

Surgical procedure for biopsies was uneventful in all cases, without immediate complaints. The day after surgery three patients were noted labial paresthesias, which disappeared in less than 1 month. One patient developed a small retention mucous cyst that disappeared spontaneously after 40 days. No patient had long-term secondary effects.

Labial salivary gland histology

All the samples collected for pathological evaluation were adequate, with more than one LSG in suitable condition for histological analysis.

In 16 individuals, the gland morphology was preserved, and there was no amyloid deposition (Figure 1). In 12 subjects, the global gland morphology was normal; however, we could observe very sparse amyloid deposits in the connective tissue, in the adventitia of the arterioles or in the proximity of the basement membrane embracing some glandular

acini (Figure 2). In 10 individuals, the global glandular aspect being preserved; we could see mild interstitial septal fibrosis and more pronounced amyloid deposition surrounding several glandular acini and excretory ducts (Figure 3). In 49 subjects, the global glandular aspect was severely changed, with evident loss of glandular acini, marked interstitial fibrosis and hyalinisation of interstitial tissue and occasional lipidic substitution with profuse amounts of amyloid (Figure 4).

The immunohistochemistry study using the antibody anti-TTR confirmed that the main component of amyloid is transthyretin in all samples with amyloid deposition. With this method, the distribution of amyloid deposits was similar to the findings obtained with Congo Red (Figure 5).

In 13 cases, the amyloid deposits were also studied by transmission electron microscopy (EM), to establish their ultrastructural characteristics. Amyloid fibrils were identified in all cases studied with EM. We have not seen significant ultrastructural changes in the glandular acini or excretory ducts cells. In the

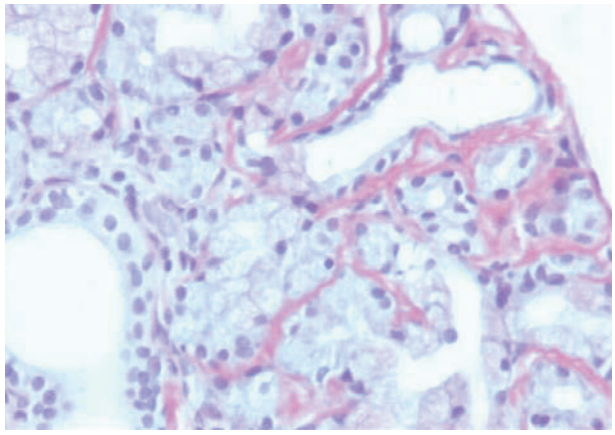

Figure 3. Congo Red × 320. Glandular acini with interticial fibrosis and evident amyloid deposition around several glandular acini.

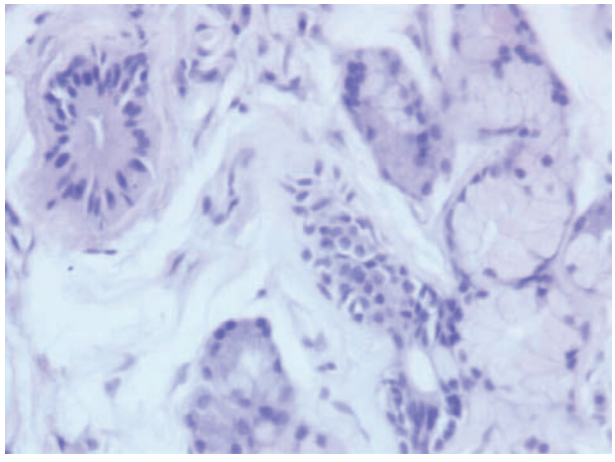

Figure 1. Congo Red × 320. Normal aspect of glandular acini and duct without amyloid deposition.

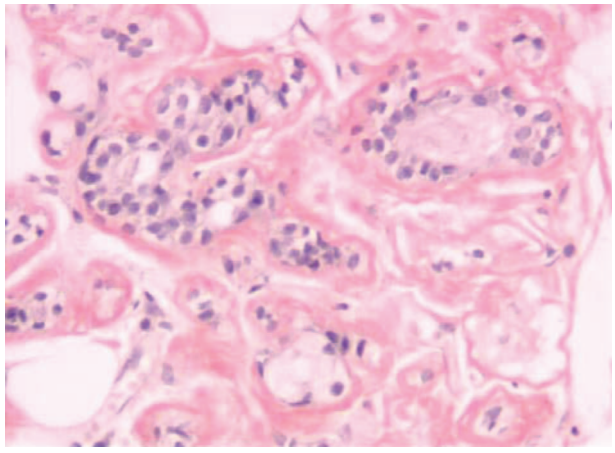

Figure 4. Congo Red × 320. Loss of glandular acini, marked fibrosis and hyalinization of interstitial tissue with very abundant amyloid deposition.

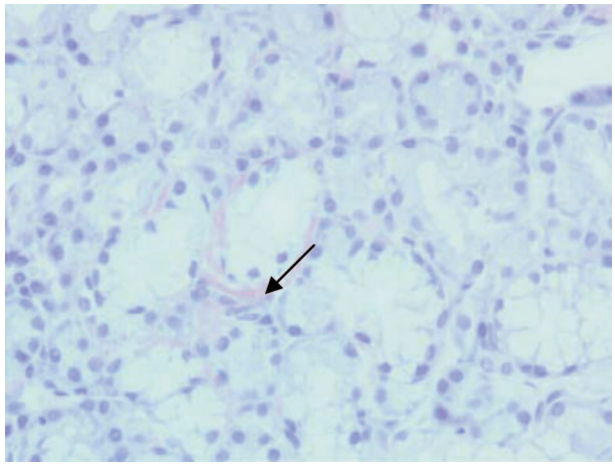

Figure 2. Congo Red × 320. Normal aspect of glandular acini, with faint deposition of amyloid around glandular acini.

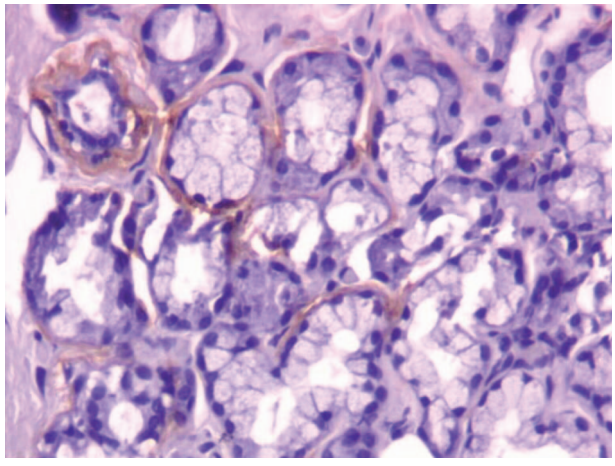

Figure 5. TTR × 500. Immunohistochemistry study using antibody against TTR. Amyloid deposits are positive for TTR.

glands with more advanced lesions, increased amounts of collagen bundles surrounding the glandular acini were seen.

The amyloid substance appeared as bundles of fine straight fibrillar material, with 8 nm of diameter, and whose size, orientation and organisation are variable (Figure 6).

This fibrillar material was among the collagen bundles but, here and there, it was also in direct contact with the basal lamina surrounding the glandular acini and the excretory ducts.

In summary, 82% of all subjects had amyloid deposition and 56% of these (49/87) (including one asymptomatic carrier) had massive amyloid deposition and significant glandular destruction (Table II).

Amyloid deposition and disease stage

The comparison between LSG histology and the clinical condition (Table III) shows that 63% of the patients (48/76) and one asymptomatic carrier have abundant amyloid deposits and gland destruction. These severe changes were found in 51% of stage 1 patients, in 64% of stage 2 patients and in all stage 3 patients. Moderate deposition of amyloid was seen in four of stage 1 patients and six of stage 2 patients. Slight amyloid deposition with normal gland morphology was restricted to stage 1 patients ($n = 11$) and one asymptomatic carrier.

Seven patients in stage 1, between the second and the fourth year of symptomatic disease, have normal biopsies, without amyloid deposition.

Statistical analysis of these data was not possible due to the small number of subjects within each subgroup.

Amyloid deposition was similar in men and in women patients ($X^2_3 = 0.936$, $p = 0.82$).

Discussion

The importance of oral manifestations of amyloidosis has been well described in AL amyloidosis, where macroglossia is considered a characteristic finding [16]. The consequences of TTR amyloidosis for the patients oral health has never been well characterised, even though xerostomia is a well-known problem of this severe autonomic neuropathy [17]. In this series of 76 patients, no significant oral problems were found and macroglossia was absent, confirming previous statements that it does not occur in TTR amyloidosis.

Table II. Distribution of histological findings in 87 subjects.

Clinical condition	Morphological classification				Total
	G0	G1	G2	G3	
Asymptomatic carriers	9	1	0	1	11
Patients	7	11	10	48	76
Total	16	12	10	49	87

Table III. Biopsy findings according to clinical stage.

Biopsy findings	Disease stage				Subjects		
	P0	P1	P2	P3	Total	Males	Females
G0	9	7	0	0	16	7	9
G1	1	11	0	0	12	7	5
G2	0	4	6	0	10	6	4
G3	1	23	11	14	49	27	22
Total	11	45	17	14	87	47	40

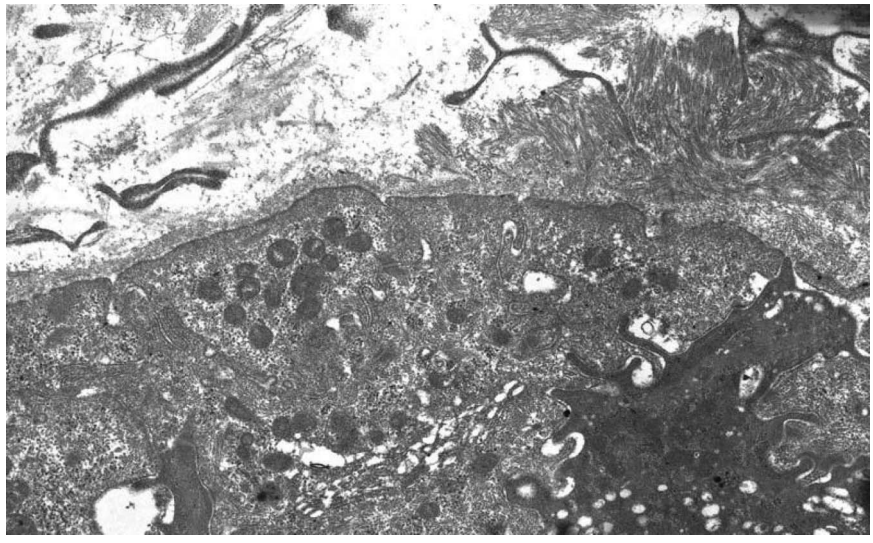

Figure 6. Electron microscopy × 12,000. Fibrillar material with the usual features of amyloid substance in contact with the right side of the basal lamina of one glandular acini.

The absence of xerostomia is more surprising and it may be explained by the absence of severely affected patients in our sample.

What causes xerostomia in TTR amyloidosis remains under discussion. The largest portion of functional saliva comes from the larger glandular structures, mainly parotid and submandibular glands. Minor salivary glands, however, have been proposed as fundamental in keeping a constant tiny film of saliva covering the oral mucosa and preventing the feeling of dryness that characterizes xerostomia [18].

The role of neuropathy – which causes denervation of glandular structures – and the role of amyloid infiltration – which causes tissue replacement with fibrosis – in the loss of saliva secretion are yet to be established. In these biopsies, we confirmed that amyloid deposition leads to significant loss of glandular tissue even in the first years of the disease, but the remaining acini and ducts have a normal ultrastructural appearance. This finding may help to explain the maintenance of a minimal saliva secretion until the late stages of the disease. Our data support previous histopathological and ultrastructural findings [19]. Functional studies of saliva secretion, which are under progress, will certainly be valuable further to clarify this subject.

FAP TTR Val 30 Met clinical onset is characterised by a sensory and autonomic neuropathy, which can be confirmed by clinical and neurophysiological exams. However, the diagnosis of amyloidosis is still based on the demonstration of amyloid deposition in the tissues. This can be proved with Congo red stain, which demonstrates the characteristic apple-green birefringence in polarised light. In our Hospital, the nerve biopsy was initially used to demonstrate amyloid deposition. Later, skin biopsy was preferred as it was an easier and less traumatic method, providing reliable results as long as sweat glands, erector pilli muscles, vessels and nerve fascicles were included in the sample [20,21]. The sampling of subcutaneous abdominal fat has been presented as the simplest form of tissue biopsy [22]. Rectal biopsy has also been advocated as a method for obtaining tissue samples for amyloid detection [23]. We believe that these methods represent procedures at least as invasive as LSG biopsy, sometimes associated with greater morbidity.

Selikoff and Robitzek [24] were the first to present evidence that gingival biopsy was of considerable value in substantiating the diagnosis of generalised secondary amyloidosis. Others argued that the gingival biopsy has little value in amyloid detection [25]. The tongue is the most frequently reported intraoral location of amyloid deposition in systemic amyloidosis [26,27]. However, the tongue biopsy is diagnostic in only 60% of cases [28]. Deep incisional gingival biopsies may cause periodontal defects. Deep incisional tongue biopsies increase the risk of damaging the neurovascular supply of this structure.

In this study, LSG biopsy was performed in all patients with only minimal and transitory morbidity and it detected amyloid deposition in 69 of 76 patients (91%). The analysis of amyloid deposition according to the clinical stage shows that all patients without detectable amyloid deposition were in the earlier stage of the disease. The irregular and patchy nature of the amyloid deposition is an acceptable explanation for this percentage of negative results, which are similar to the findings of other series, regardless of the tissue studied. It must be underlined that 100% of patients with moderate disease have a positive biopsy.

The finding that, among 11 asymptomatic carriers, one had light amyloid deposits and another had moderate amyloid deposits and some degree of glandular destruction indicates that the pathogenic process starts (and may even progress significantly) before clinical symptoms appear.

We conclude that LSG biopsy is a simple procedure, resulting in a quicker healing time and increased patient comfort, with a very good sensitivity. It should be considered as a method of choice for the diagnosis of TTR amyloidosis, before liver transplant or in patients included in experimental therapeutic studies.

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References

1. Andrade C. A peculiar form of peripheral neuropathy. Familial atypical generalized amyloidosis with special involvement of the peripheral nerves. Brain 1952;75:408–427.
2. Sousa A, Coelho T, Morgado R, Coutinho P. Statistical analysis of factors which may influence the duration of FAP type I. In: Costa PP, Freitas AF, Saraiva MJM, editors. Familial amyloidotic polyneuropathy and other transthyretin related disorders. Porto: Arquivos de Medicina; 1990. pp 351–355.
3. Lobato L, Beirão I, Silva M, Bravo F, Silvestre F, Guimarães S, Sousa A, Noel LH, Sequeiros J. Familial ATTR amyloidosis: microalbuminuria as a predictor of symptomatic disease and clinical nephropathy. Nephrol Dial Transplant 2003;18:532–538.
4. Sandgren O, Drugge U, Holmgren G, Sousa A. Vitreous involvement in familial amyloidotic polyneuropathy: a genealogical and genetic study. Clin Genet 1991;40:452–460.
5. Fonseca C, Ceia F, Carvalho A, Nogueira JS, Morais H, Conceição I, Sales Luis ML, Sales Luis AS. The natural

history of cardiac involvement in Portuguese-type familial amyloid polyneuropathy. Rev Port Cardiol 1997;16:101–105.
6. Planté-Bordeneuve V, Lalu T, Misrahi M, Reilly MM, Adams D, Lacroix C, Said G. Genotypic-phenotypic variations in a series of 65 patients with familial amyloid polyneuropathy. Neurology 1998;51:708–714.
7. Benson MD. The molecular biology and clinical features of amyloid neuropathy. Muscle & Nerve 2007;36:411–552.
8. Saraiva MJM, Costa PP, Goodman DS. Genetic expression of a transthyretin mutation in typical and late-onset Portuguese families with familial amyloidotic polyneuropathy. Neurology 1986;36:1413–1417.
9. Nakazato M, Steen L, Holmgren G, Matsukura S, Kangawa K, Matsuo H. Structurally abnormal transthyretin causing familial amyloid polyneuropathy in Sweden. Clin Chem Acta 1987;167:341–342.
10. Ikeda S-I, Nakazato M, Ando Y, Sobue G. Familial transthyretin-type amyloid polyneuropathy in Japan clinical and genetic heterogeneity. Neurology 2002;58:1001–1007.
11. Munar-Qués M, Maria JM Saraiva; Viader-Farré C, Zabay-Becerril JM, Mulet-Ferrer J. Genetic epidemiology of familial amyloid polyneuropathy in Balearic Islands (Spain). Amyloid 2005;12:54–61.
12. Lechapt-Zalcman E, Authier FJ, Creance A, Voisin MC, Gherardi RK. Labial salivary biopsy for diagnosis of amyloid polyneuropathy. Muscle & Nerve 1999;22:105–107.
13. Hachulla E, Grateau G. Diagnostic tools for amyloidosis. Joint Bone Spine 2002;54:538–545.
14. Coutinho P, Silva AM, Lima LJ. Forty years of experience with type I amyloid neuropathy. Review of 483 cases. In: Glenner GG, Costa PP, Freitas AF, editors. Amyloid and amyloidosis. Amsterdam: Excerpta Medica; 1980. pp 88–98.
15. Sousa A, Coelho T, Barros J, Sequeiros J. Genetic epidemiology of familial amyloidotic polyneuropathy (FAP) - type I in Póvoa do Varzim/Vila do Conde (North of Portugal). Am J Med Gen (Neurop Genetics) 1995;60:512–521.
16. Falk RH, Comenzo RL, Skinner M. The systemic amyloidosis. N Engl J Med 1997;337:898–909.
17. von Bültzingslöwen I, Sollecito TP, Fox PC, Daniels T, Jonsson R, Lockhart PB, Wray D, Brennan TM, Carrozzo M, Gandera B, Fujibayashi T, Navazesh M, Rhodus NL, Schiøt M. Salivary dysfunction associated with systemic diseases: systematic review and clinical management recommendations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;103(S1):S57–S65.
18. Eliasson l, Birkhed D, Carlén A. Feeling of dry mouth in relation to whole and minor saliva secretion rate. Arch Oral Biol 2009;54:263–267.
19. Delgado W, Arana-Chavez A, Victor E. Amyloid deposits in labial salivary glands identified by electron microscopy. J Oral Pathol Med 1997;26:51–52.
20. Guimarães A, Pinheiro AV, Leite I. Sural nerve biopsy in familial amyloidotic polyneuropathy: a morphological and morphometric polyneuropathy. In: Isobe T, Araki A, Uchino F, Kito S, Tsubura E, editors. Amyloid and amyloidosis. New York: Plenum Press; 1988. pp 493–498.
21. Gabriel CM, Howard R, Kinsella N, Lucas S, McColl I, Saldanha G, Hall SM, Hughes RAC. Prospective study of 4 the usefulness of sural nerve biopsy. J Neurol Neurosurg Psychiatry 2000;69:442–446.
22. Haagsma EB, Van Gameren II, Bijzet J, Posthumus MD, Hazemberg BPC. Familial amyloidotic polyneuropathy: long-term follow-up of abdominal fat tissue aspirate in patients with and without liver transplantation. Amyloid 2007;14:221–226.
23. Kyle RA, Gertz MA. Primary systemic amyloidosis: clinical and laboratory features in 474 cases. Semin Hematol 1995;32:45–59.
24. Selikoff IJ, Robitzek EH. Gingival biopsy for the diagnosis of generalized amyloidosis. Am J Pathol 1947;23:1099–1111.
25. Lovett DW, Cross KR, Van Allen M. The prevalence of amyloid in gingival tissue. Oral Surg Oral Med Oral Pathol 1965;20:444–448.
26. Salisbury PL, Jakoway JR. Oral amyloidosis: a late complication of multiple mieloma. Oral Surg Oral Med Oral Pathol 1983;56:48–50.
27. Biewend ML, Menke DM, Calamia KT. The spectrum of localizes amyloidosis: a case series of 20 patients and review of the literature. Amyloid 2006;13:135–142.
28. Pribitkin E, Friedman O, O'Hara B, Cunnane MF, Rosen M, Keane WM, Sataloff RT. Amyloidosis of the upper aerodigestive tract. Laryngoscope 2003;113:2095–2101.

IV. SIALOMETRY AN SIALOCHEMISTRY IN FAMILIAL AMYLOIDOTIC POLINEUROPATHY TTRVal30Met

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Abstract

Background: Dysfunction of salivary glands is assessed by measuring salivary flow rate and by chemical analysis for which whole saliva is most frequently used. Sialochemistry has been proposed as a tool for the differential diagnosis of various salivary gland diseases, including Sjögren Syndrome, as many salivary gland diseases are well documented in the literature with regard to their sialochemical manifestations.

Salivary hypofunction has been described in FAP Sweden patients by Johansson et al in 1992.

Objective: To define salivary hypofunction and several salivary parameters in FAP Portuguese patients and comparing them with those obtained by Johansson et al in 1992.

Methods: Sialometry and sialochemistry were done in 39 subjects with molecular diagnosis of TTRVal30Met mutation. They were all submitted to a stomatological and neurological observation to evaluate oral health problems and to determine the neurological stage of the disease. The mean duration of symptomatic disease was 4.1years (±2.6SD).

Results: There were statistically significant differences in salivary flow rate between FAP patients in early stage of the disease and in patients with more advanced disease (9.320 cm in FAP0 and 3.450 cm in FAP3). The concentration in protein, glucose, potassium, cholesterol, lysozyme, bicarbonates, IgA and pH were higher in the familial amyloidotic polyneuropathy patients than in the control group.

Conclusions: In FAP Portuguese patients there are a reduced saliva secretion and some salivary parameters are different than in the control group.
This is a preliminary study so the results must be interpreted with caution.

Introduction

Normal salivary gland function is considered to be critical for the maintenance of a healthy oral mucosa. Saliva provides protection to its cleaning, lubricating and antimicrobial properties.

The salivary composition is modified as it moves from the acinar region to the oral cavity. Proteins are produced and added to the saliva from both acinar and ductal cells. In the ductal region, sodium and chloride are absorbed while potassium is secreted, which results in a final hypotonic, protein-rich saliva as it enters the oral cavity. Normal basal (resting) salivary secretion is basically hypotonic, slightly alkaline and rich in potassium, whereas stimulated saliva is less hypotonic and has a higher concentration of sodium and chloride, with a lower concentration of potassium (Dawes C 1987).

Salivary dysfunction results from qualitative or quantitative alteration in the output of saliva caused by increased (hyperfunction) or decreased (hypofunction) in salivary flow (Millard HD, Mason DK 1998).

Hypersalivation, or sialorrhea, is defined as excess salivary flow. It can be related to conditions such as neurological disorders (e.g., Parkinson, Down syndrome, amyotrophic lateral sclerosis, cerebral palsy, autism, Fragile X). Furthermore, drugs that have a cholinergic effect may cause hypersalivation (Boyce HW and Bakheet MR 2005). Drooling, which can sometimes be a manifestation of hypersalivation, is not always attributable to excess salivary output but rather caused by inability to retain and swallow saliva because of weakness in perioral muscle tonus or dysphagia.

The terms salivary hypofunction or hyposalivation and xerostomia are often incorrectly used. Hyposalivation refers to decreased salivary flow, whereas xerostomia refers to a subjective experience of mouth dryness. This is further complicated by the fact that some patients with hyposalivation are not xerostomic and, conversely, those with xerostomia may have normal salivary flow rates.

Xerostomia, the perception or symptoms of dry mouth, is usually caused by a decrease of at least 50% in unstimulated salivary flow rate (Dawes C 1987), and can be accompanied by oral soreness (Navazesh et al 1992) and burning sensations (Pedersen AM et al 2002).

Although there are extensive individual variations, hyposalivation is usually defined as an unstimulated whole saliva flow rate of less than 0.1 mL/min,

collected for 5 to 15 minutes, or chewing-stimulated whole saliva flow rate less than 0.7mL/min, collected for 5 minutes.

In contrast to hypersalivation, hyposalivation may present with clinically significant secondary problems such as mucosal infections, dental caries of cervical, incisal surfaces or cusp tips, and difficulties to swallow, taste, eat and speak.

Proposed mechanisms for hyposalivation include neurotransmitter receptor dysfunction, salivary gland parenchymal destruction, immune deregulation that may interfere with secretory processes, radiation-induced cellular DNA damage, alterations of fluid and electrolytes and combination of some of them.

Considering all these variations, it is important to remind that there are various systemic diseases that have an effect on salivary function, causing short and long-term sequelae.

A number of genetic or other chronic diseases may be associated with salivary dysfunction such as, diabetes mellitus, autoimmune thyroiditis, chronic pancreatitis, celiac disease cystic fibrosis, familial amyloidotic polyneuropathy myotonic dystrophy, Papillon-Lefèvre syndrome Prader-Willi syndrome, sphingolipid storage disease, Gaucher disease, and systemic thalassemia major. A systematic revision of these aspects was made by von Bültzingslöwen et al in 2007.

Various diseases and conditions causing metabolic changes are associated with salivary hypofunction such as dehydration (Ship JA et al 1997), eating disorders (anorexia/bulimia) (Riad M et al 1991), end-stage renal disease (Bots CP et al 2005) and nutritional deficiencies (Flink H et al 2000).

Sjögren syndrome (SS) is one of the most frequently encountered chronic, autoimmune, inflammatory connective tissue disorders, being women the majority of patients affected, encompassing 90% of all cases - it has been considered as a paradigm of salivary dysfunction (Gannot G et al 2000). SS can be seen alone (primary SS) or in association with another autoimmune rheumatic disease (secondary SS), usually rheumatoid arthritis, but also including systemic lupus erythematosus or another autoimmune connective tissue disorder. The pathogenesis of SS is still obscure as the disease is multifactorial and complex.

Systemic amyloidosis (Gertz MA et al 2005) and familial amyloidotic polyneuropathy have proven to be two pathological conditions that occur with changes in the saliva composition (Johansson I et al 1992).

The aim of the present investigation is to study, in FAP Portuguese patients, saliva secretion rate and composition and other factors related to the risk of development of oral disorders, such as periodontitis, dental caries and the relationship between some salivary parameters, disease staging and salivary gland histology.

Material and Methods

Subjects

The present investigation was conducted in 39 individuals with Familial Amyloidotic Polyneuropathy (FAP), including 14 males and 25 females with a mean age ±SD 35.33±10.42 years. The mean duration of symptomatic disease was of 4.1±2.6 years [1-14]. Median was 3 years and mode was 2 years. The control group was constituted by 37 individuals, including 18 males and 19 females with a mean age ±SD 38.32±10.64.Patients in P4 disease staging there are not includes in this investigation.

All patients had TTRVal30Met mutation determined by DNA analysis and a neurological observation to establish the medical history and define the clinical stage (Coutinho P et al 1980).

There were no complaints of xerostomia or difficulties in eating, swallowing, tasting or speaking. We have not seen any patient with candidiasis or atypical dental caries (in cervical, incisal surfaces or cusp tip locations). Some patients had tooth loss, and marginal periodontitis caused by dental plaque. There was no clinical evidence of amyloid deposition in the oral cavity, namely the presence of macroglossia.

All the individuals filled a questionnaire including: age, weight, number of pregnancies and children, medical history and hospitalizations, current treatment, smoking or alcohol habits, number of teeth and usage of xerogenic drugs, such as antihypertensive, β blockers, antihistamines and psychotropic.

The Ethical Committee of Hospital de Santo António approved this study and all subjects signed an informed consent form before the study procedures.

Collection of saliva samples

The collection of stimulated whole saliva was performed under resting between 10:00 and 12:00 am. Preliminary instructions were supplied to each individual and they were warned not to ingest any type of foods or liquids, brush the teeth, and chew chewing gums or candies two hours before the saliva collection.

Before beginning the evaluation, the individuals swallowed all the saliva they had in their mouths at that moment.

During the five minutes of the measurement, the individuals sat down in a chair, bending over with their elbows rested on the thighs, in such a way that the head was tilted forward. In these positions they were relaxed, closing the eyes and breathing by the nose. Clinical data and the environmental conditions were registered.

To collect saliva, a standardized tube with two compartments and standardized cotton were used. Both the cotton and two-compartment tube were obtained from the same manufacturer (Salivette, Sarstedt, Nümbrecht, Germany). The upper part of the tube containing the cotton presented a hole, so that after centrifugation, the saliva was recovered in the lower part and became available for analysis.

Centrifugation of the device at 3000 g for 10 minute allowed the saliva adsorbed to the cotton to pass through the orifice into the lower compartment of the device, being the saliva immediately frozen at – 80º C.

When compared with the classic methods of saliva collection, namely the paraffin test, this method is reliable, but we must be careful in the interpretation of some salivary parameters. The values obtained with this method cannot be compared with the values of reference normally used and a control group had to be used (Lenander-Lumikari M et al 1995).

Salivary flow rate

The method chosen to determine the salivary flow was developed by Lopez-Jornet in 1994 and it is similar to the Shirmer test used to measure the lacrimal secretion. It is performed using a strip of millimetric paper (paper Whatman degree 41, with quick filtration and with an elevated degree of purity,

as well as an elevated resistance to rupture, when wet) with one centimeter of width and seventeen centimeters of length introduced in a polyethylene bag.

To carry out the test, the first centimeter of the strip, which is not graduated, is doubled in 90° and it is introduced in the oral cavity under the tongue. The lips are in contact with the polyethylene bag, which protects the millimetric scale. In this way, most of the plastic bag that contains the strip is outside the mouth and is soaked progressively by the saliva. After five minutes the wet millimeters are measured.

Laboratory assays

Salivary immunoglobulins A, G and M concentrations were analyzed using a nephelometric assay (IMMAGE 800 Immunochemistry System, Beckman Coulter). Lysozyme was assessed by a turbidimetric method with the FAR diagnostic kit (Cobas Mira Pus-ABX reagent) and pH was assessed using the test strip t 6.50-10.00 of Merck. Specific gravity was performed with a Laboratory Densimeter (Atago Company). Osmolarity was assessed by osmometry (Osmometer Model 3320- Advanced Instruments, Inc.). Uric acid was measured with the colorimetric enzyme method with uricase (Cobas Integra 800, Roche). Amylase was analyzed using an enzymatic colorimetric test kit with a dilution of 1/100 (Cobas Integra, Roche). Glucose was measured with the colorimetric enzyme method with hexokinase (Cobas Integra, Roche). Urea was measured by a kinetic method with urease and glutamate dehydrogenase (Cobas Integra 800, Roche). Creatinine was assessed by the kinetic Jaffe method without deproteinization (Cobas Integra 800, Roche). Bicarbonates were analyzed by an enzymatic method with phosphoenolpyruvate carboxylase and malate dehydrogenase (Cobas Integra, Roche). Ammonia was measured by an enzymatic method using glutamate dehydrogenase with a dilution of 1/10 (Cobra Integra, Roche). Albumin was assessed by an immunoturbidimetric method (Cobra Integra, Roche). Total protein was measured with the pyrogallol molybdate red complex colorimetric method (Cobras Integra, Roche). Cholesterol was assessed by a colorimetric method (Cobas Integra, Roche). Calcium was measured by the cresolftaleine method (Cobas Integra, Roche). Phosphate was measured by the phosphomolybdate direct method (Cobra Integra, Roche). Magnesium, sodium, potassium and chloride were assessed by de ion selective electrode method (Cobra Integra, Roche).

Statistical analysis

Student's t-tests or the Mann-Whitney U tests were used to study the quantitative variables between two independent samples. The choice of parametric or non-parametric test depended on whether or not the values of quantitative variables analyzed presented a normal distribution, which was determined using the Kolmogoronov-Smirnov test.

The Pearson coefficient correlation was used to study the correlation between quantitative variables. In those cases with a significant correlation, the equation of the simple regression model was determined. Statistical significance was taken as a value of $p<0.05$.The results were analyzed using the SPSS version 15.0 statistical package for Windows (SPSS Inc. Chicago, USA).

Results

The salivary parameters studied are listed in Table 1. In this study there were no differences between the FAP patients and control group concerning tobacco, medicamentous and alcoholic habits. This sample had a larger number of women than men, which may be explained by the fact that women are looking earlier for medical care. The body mass index for FAP patients was 24.08 and for control group was 25.7. For sodium, lysozyme, IgG and IgM we found some lower values to the inferior limit of the method, so we divided it in two categories. As for the sodium, one category had values lower than 21 mmol/L and another one with values equal or superior to 21 mmol/L. In two groups all the equal or superior values of sodium to 21mmol/L were found in the FAP group ($p<0.02$). The IgG was divided in two categories: one had values lower than 0.9 UI/L and another one with values equal or superior to 0.9 UI/L. The differences were not statistically significant between the two groups. Lysozyme was divided in three categories: one with lower values than 6 mg/L, another with values between 6.0 and 12.0 mg/L and the third one with values superior to 12.0 mg/L. The values between 6.0 mg/L and 12.0 mg/L and inferior to 6.0 mg/L are more frequent in FAP group.

Salivary parameters	FAP patients	Control group	p
Albumin (mg/L)	37.95(7.5-419.6)	25.30(6.30-1239.6)	0.03
Amylase (UL)	82850 (4406-725800)	73300 (2300-678000)	0.94
Glucose (mg/dL)	5.5 (0-31.0)	3.0 (1.0-11.0)	0.00
Creatinine (mg/dL)	0.2 (0.0-2.0)	0.2 (0.0-2.0)	0.86
Calcium (mmol/L)	2.09 (1.0-4.0)	2.04 (1.2-5.0)	0.68
Phosphate(mmol/L)	5.11 (3.3.14.1)	5.23 (2.1-12.6)	0.87
Potassium (mmol/L)	21.0 (14.2-43.2)	20.2 (14.6-30.0)	0.19
Chloride (mmol/L)	21.5 (15.0-42.0)	23.0 (20.0-44.0)	0.94
Protein (g/L)	0.42 (0.10-0.73)	0.37 (0.10-2.00)	0.03
Cholesterol (mg/L)	0.0 (0.0-2.5)	0.0 (0.0-1.00)	0.01
Lysozyme (mg/L)	2.0 (2.0-30.0)	2.00 (0.90-17.4)	0-16
Bicarbonates (mmol/L)	0.00 (0.00-750)	0.70 (0.00-11.60)	0.00
IgA (UI/mL)	8.40 (3.00-47.50)	7.80 (3.30-31.90)	0.05
Osmolarity (mosmol/Kg)	65.5 (36.0-134.0)	60.0 (40.0-102.0)	0.25
pH	6.50 (6.00-8.10)	6.00 (5-8.30)	0.00
Specific gravity	1.001 (1.000-1.006)	1.001 (1.000-1.005)	0.13
Ammonia (mmol/L)	3500 (270-70000)	3751 (260-70000)	0.19

Table 1. Salivary parameter analyzed between two groups

Salivary Flow rate

Table 2 presents the relationship between salivary flow rate and disease staging.

The mean flow rate was 6. 45±2, 83 cm in FAP patients and 7. 22±3.03 cm in the control group are not statistically significant different (p =0.26).

Staging (P)	Salivary flow rate (mean values)	FAP Patients (n)
P0	9.320 cm	5
P1	6.413 cm	23
P2	6.230 cm	7
P3	3.450 cm	4

Table 2. Distribution of the salivary flow according to the disease stage

Buffering capacity

The bicarbonates values were higher in the control group being statistically significant different (p=0.00). Concerning the proteins the highest values were found in the patients with FAP being this statistically significant different (p=0. 03). Albumin particularly presents statistically significant higher values (p=0.03) in the FAP group of patients. We did not find statistically significant differences in the phosphates values in both studied groups.

A statistically significant (p=0.00) positive correlation was found between the bicarbonates and the pH. As the bicarbonates increases the salivary pH becomes more alkaline. We found a statistically significant difference for the pH in two groups (p=0.00).

In the group of FAP patients we found a positive and statistically significant correlation (p=0.00) between the proteins and the osmolarity.

The mean salivary concentration of ammonia was similar in both groups 3500 mmol/L [270-70000] and 3751mmol/L [260-10300] respectively. A statistically significant (p=0.01) positive correlation was found between ammonia and the CPO index in the group of FAP patients. The mean salivary concentration of urea is similar in both groups 31.6 mmol/L [0-67.0] and 32.32 mmol/L [16.0-62.0] respectively.

Electrolytes

Sodium values lower than 21mmol/L was found in both groups. Equal or superior values of sodium to 21mmol/L were found in the FAP group with a statistically significant difference ($p<0.02$).

Calcium values were similar for FAP group and control group 2.09 mmol/L [1.0-4.0] and 2.04 mmol/L [1.20-5.0]) respectively. In both groups the potassium values found were similar, 21.0 mmol/l [14.2-43.2] for the FAP patients and 20.2 mmol/L [14.6-30.0]) for the control group.

There were no statistically significant differences in chloride 21.0 mmol/L [15.0-42.0] for the FAP patients and 23.0 mmol/L [20.0-42.0]), phosphate 5.11mmol/L [3.3-14.1] for the FAP patients and 5.23 mmol/L [2.1-12.6]) in both groups.

The mean salivary pH values were higher in the control group than in FAP group 6.50 [6.00-8.01]) for the FAP patients and 6.00 [5.00-8.30]) respectively, and are statistically significant differences ($p=0.00$).

Discussion

Xerostomia is the most common symptom in Sjögren syndrome. It is caused by a decrease in salivary flow rate and there are also differences in protein content of saliva, including a decrease in secretory IgA, which increases the antibacterial defense system against caries (Cuida M et al 1997).

As in Sjögren syndrome, the secretion and quality of saliva has been studied in FAP patients, in whom xerostomia is present in later stages of the disease (Johansson et al in 1992).

Our present study shows a decrease in salivary flow in FAP patients, also showing that is correlated to the progression of the disease (Table 2). In fact, there is less production of whole saliva in patients with a greater deposition of amyloid in the salivary glands. These results are similar with those reported by Johansson et al in 1992.

The sample studied by one investigator reports a mean age of 60.2 years±10.3 years. In our sample the mean age is lower (35.33±10.42 years).

In our sample, when we analyze the disease stage as a covariate factor, significant differences are found between the extremes ($p=0,01$), with 4 patients in the stage 3 with lower mean values of salivary flow and 5 asymptomatic

patients with higher mean values of salivary flow. However, our results should be carefully analyzed because the type of saliva sample was different (stimulated saliva in our cases), as well as the mean age of the patients.

Age influences salivary secretion, probably due to the physiologic process of ageing (Lopez-Jornet MP et al 1994). In our sample there were no differences between FAP patients and the control group. We can explain this fact as the mean age of the two samples is very similar.

When we compare others parameters such as body mass index, age, sex, temperature, humidity, CPO index, pH and its influence in salivary flow rate, only the body mass index shows statistically significant different for male sex and FAP patients ($p = 0.00$). For control group that difference was not clear ($p = 0.07$).

In Portuguese FAP patients there is a decrease in salivary flow that is more evident in advanced disease (FAP 3). Secretory IgA presents higher values in FAP patients, being statistically significant different ($p=0.05$). This fact suggests a protective effect causing the lower incidence of caries and other pathologies, mainly oral candidiasis, observed in this patient.

In FAP more advanced stage (FAP3), the global glandular aspect of labial salivary glands was severely changed, with evident loss of glandular acini, marked interstitial fibrosis of interstitial tissue and occasional lipidic substitution with profuse amounts of amyloid. This fact can explain the decrease in salivary flow (Do Amaral Barbas et al 2009).

FAP patients present an increase in glycosylation and the water content of saliva is reduced, whereas the protein secretion is increased (Monteiro FA et al 2006).

In this study the mean protein concentration in FAP patients is higher than in the control group and this difference is statistically significant ($p=0.03$). Albumin particularly presents statistically significant with higher values ($p=0.03$) in the FAP patients. Albumin may act as a blocking agent in the complex process of Candida albicans adhesion (Bürgers R et al 2010), that could explain the absence of oral candidiasis in FAP patients sample.

Whole saliva contains three major systems contributing to the buffer capacity: the bicarbonate, the phosphate, and the protein buffer systems. However, their concentrations and ability to buffer are dependent of salivary flow rate (Tenovuo J 1997).

In the group of FAP patients we found a positive and statistically significant correlation ($p=0.00$) between the proteins and the osmolarity. Together with the

bicarbonates, they contribute to a regulatory effect on the pH providing to saliva alkalinity. We did not find a positive correlation between the salivary flow rate and the pH.

The alkaline pH, in association with the higher content in albumin, could explain the low CPO index of the FAP patients when compared with the control group.

Urea enters the oral cavity in all salivary secretions and in the gingival crevicular fluid at concentrations ranging between 1 and 10 mmol in healthy individuals (Golub I et al 1971; Kopstein J and Wrong OM 1977). The hydrolysis of urea by bacterial urease enzymes generates ammonia and CO2 and it is considered a major pathway for alkali production in the oral cavity (Sissons CH and Cutress TW 1988). Numerous studies have shown that urea at concentrations comparable to those normally found in saliva can significantly increase the baseline pH of dental plaque. It can effectively counteract the effects of glycolytic acidification on the plaque pH (Dawes C and Dibdin GH 2001). Therefore, it has been hypothesized that the production of ammonia via ureolysis in the oral cavity may be an important factor inhibiting the emergence of a cariogenic flora and the development of caries. Indeed, a link between markedly elevated urea levels in the saliva of renal dialysis patients and caries resistance has been noted (Nandan RK et al 2005).

The two groups studied (FAP patients and control group) presented higher values of amylase and ammonia. We looked for a correlation between the values of ammonia and the CPO index. In the group of healthy volunteers this correlation does not exist. As for the group of the FAP patients, it is positive and statistically significant ($p = 0.01$). The above mentioned factors could explain the low CPO index of the FAP patients when compared with the control group.

Among others salivary parameters, alpha-amylase has been correlated with clinical parameters of periodontal disease (Rai B et al 2011). In this study we do not found statistically significant differences in amylase between two groups that could explain the absence of periodontal disease in FAP patients and in the control group.

The lysozyme and peroxidase system are the two main enzymes of the immune system of the oral cavity defense. The antimicrobial activity of lysozyme occurs through a muramidase-dependent mode and a cationic-dependent or structure-related bactericidal mechanism (Ibrahim HR et al 2001). The antifungal activity of lysozyme and the peroxidase system have been reported (Lee JY et al

2010). Concerning the lysozyme, there are not statistically significant differences between two groups. Although the group of the FAP patients presents the higher values, this may explain some protection to oral candidiasis.

Soares MS et al in 2009 had measured the concentration of salivary glucose in 63 healthy patients (non-diabetic and without oral pathology) and found a mean value of 5.94 mg/dl that are similar to the measurement of 5.57 mg/dl recorded by Di Gioia ML et al in 2004.

In this present study we found a mean value of 5.5 mg/dl of glucose in FAP patients that is higher than the values recorded in control group, being this difference statistically significant ($p=0.00$). Comparing our data with those obtained by Soares MS, our glucose values are slightly different. One explanation for these differences may be the choice of the study designs, as well as the diversity of methods and criteria for selecting the sample and the method to collect saliva.

Several studies were made in Sjögren patients that show higher values of sodium and chloride in whole saliva whether stimulated or not (Kalk WW et al 2002; Pijpe J et al 2007). The concentrations of sodium and chloride have also been shown to be higher in submandibular and sublingual glands of patients with primary SS and secondary SS compared to patients with clinical conditions resembling SS, as sialadenosis, sodium retention dysfunction syndrome and medication-induced xerostomia (Kalk WW et al 2002).

In FAP patients we found equal or superior values of sodium to 21mmol/L with a statistically significant difference ($p<0.02$). This fact should be interpreted with caution, because the composition of the final saliva secreted in oral cavity strongly depends on the secretion rate in such a way that at low flow rates the saliva contains low sodium and chloride and as the flow rate increase, the concentration of sodium and chloride will raise.

The lipid content and composition of parotid and submandibular saliva from caries-resistant and caries-susceptible adults was investigated. That data suggest a relationship between the level of lipids in salivary secretions and resistance to caries (Slomiany BL et al 1982). In this present study higher values of cholesterol were found in FAP patients and there is a statistically significant difference ($p = 0.01$). These data are similar to those collected by Slomiany BL et al. However, they should be considered with caution because both the methodology used to collect saliva and sample sizes are different.

The present study shows that there are differences in some salivary parameters (glucose, urea, cholesterol, proteins, albumin in particular and salivary flow rate) between the FAP patients and the control group. Besides, salivary flow rate is lower in more advanced stage disease, which is similar with data founded in the Johansson study. However, this is a preliminary study, whose findings should be viewed with caution and should be pursued involving a larger number of patients in different stages of the disease.

V. SALIVARY BIOMARKERS

V.A. MMP-9 levels in labial salivary glands and saliva correlate with amyloid deposition in TTTRVal30Met carriers.

Contributors to this paper:

Dras. Tania Ribeiro[1] and Diana Martins[1] in Immunocytochemistry and ELISA processing samples.

Dr. Antonio Guimarães[2] in histological interpretation, analysis and discussion of the results.

Abstract

Background: Familial amyloidotic polyneuropathy (FAP) is a disorder characterized by extracellular deposition of fibrillar transthyretin as amyloid, with a special involvement of the peripheral nerves. Several extracellular matrix proteins have been found elevated in tissues from FAP patients, mainly metalloproteinase-9 (MMP-9), neutrophil gelatinase associated lipocalin (NGAL) and byglican.

Objective: To evaluate the levels of MMP-9 in labial salivary glands (LSG) and saliva from FAP patients at different stages of the disease.

Methods: Labial salivary glands were obtained from 52 patients (21 males, 31 females) and 10 control subjects (4 males, 6 females). Saliva sample were collected from 68 patients (30 males, 38 females) and 9 control subjects (2 males, 7 females). All patients were diagnosed for TTRVal30Met mutation by DNA analysis and submitted to a neurological observation to determine the neurological stage of the disease. Ten patients (3 males, 7 females) had liver transplant. The mean time of transplant was 32.8 months (±24.1 SD).

Results: ANOVA show statistically significant differences in MMP-9 LSG levels in patients at different disease stages when compared with control group. No statistically significant differences were found between MMP-9 salivary levels and amyloid deposition.

Conclusions: The MMP-9 levels in LSG increase with the severity of the disease revealing a positive correlation between MMP-9 levels and disease progression.

Introduction

Familial amyloid polyneuropathy (FAP) is a neurodegenerative autosomal dominant disorder characterized by the systemic extracellular deposition of mutated transthyretin amyloid fibrils in several tissues, particularly in the peripheral nervous system (Andrade C 1952). The most common point mutation in TTR is a substitution of valine for methionine at position 30 of the polypeptide chain, which promotes amyloidogenesis (Saraiva MJ et al 1983). In amyloid related-disorders, amyloid deposits are composed of two types of components: the disease-specific peptide/protein and amyloid-associated molecules that include serum amyloid P component (Pepys MB et al 1994), apolipoprotein E (Gallo G et al 1994) and extracellular-matrix related components such as proteoglycans (Lyon A et al 1991). Changes in proteoglycans type and distribution could account for the derangement of collagen and for the alterations of physical properties of tissues with TTR deposition. Remodeling of the extracellular matrix occurs continuously and is affected by various cytokines (Tufvesson E et al 2000).

Metalloproteinases are a large family of important proteases that include matrix metalloproteinases and proteins with a disintegrin and metalloproteinase domain (Cauwe B et al 2007), and alterations of their levels are a hallmark of many inflammatory processes leading to irreversible alterations in tissue architecture (Stamenkovic I 2003).

Oral manifestations of amyloidosis have been particularly described in AL amyloidosis, where macroglossia is considered a characteristic finding (Falk RH et al 1997). However, the consequence of TTR amyloidosis for the patient's oral health has never been well characterized, even though xerostomia is a common problem of this severe autonomic neuropathy (von Bültzingslöwen et al 2007). Juusela P et al in 2009 presented a case of sicca syndrome, originally diagnosed as primary Sjögren's syndrome but later found to represent an initial disease manifestation of AGel amyloidosis, not recognized earlier.

The role of neuropathy – which causes denervation of glandular structures – and the role of amyloid infiltration – which causes tissue replacement with fibrosis – in the loss of saliva secretion are yet to be established. There are some FAP patients that had normal labial salivary glands despite the amount of amyloid deposition. In a vast majority of patients

amyloid deposition leads to significant loss of glandular tissue even in the first years of the disease, but the remaining acini and ducts have a normal ultrastructural appearance. In advanced disease staging there are marked interstitial fibrosis and hyalinization of interstitial tissue and occasional lipidic substitution with profuse amounts of amyloid (Do Amaral Barbas et al 2009).

In salivary glands, the basal lamina in acini is connected to the cytoskeleton of acinar cells via integrins in the basal plasma membrane (Yeaman C et al 1999). Since specific matrix metalloproteinases (MMPs) in particular MMP-2, MMP-3, MMP-9 degrade basal lamina components and since patients with primary SS show elevated levels of MMP-9 in saliva it has been hypothesized that MMPs might be involved in loss of cell anchorage to the basal lamina (Konttinen YT et al 1998).

Primary Sjögren's syndrome (SS) is an autoimmune exocrinopathy of unknown etiology in which progressive replacement of exocrine components by fibrous and adipose tissue leads to symptoms such as oral and ocular dryness (Skopouli FN et al 1998). Important changes take place in the structure and function of the parenchyma and extracellular matrix of salivary glands of SS resulting in glandular atrophy in patients with severe disease (Goicovich et al 2003).

MMP-9 occurs as a complex with neutrophil gelatinase-associated lipocalin (NGAL); both molecules were found increased in FAP and in vitro degraded TTR aggregates and fibrils. Byglican, NGAL and MMP-9 are transcriptionally up-regulated by NF-kB, a transcription factor that is activated in FAP nerves and salivary glands (Sousa MM et al 2004).

The aim of this study was to investigate if the levels of MMP-9 in labial salivary glands and saliva could explain the changes in salivary gland histology as a possible co-factor related to amyloid deposition, and whether could serve as a biomarker to distinguish precocious stages of the disease and correlate with disease progression.

Material and methods

Labial salivary glands (LSG) biopsies

Labial salivary glands (LSG) biopsies were obtained from 52 individuals (21 men, 31 women) and 10 control subjects (4 men, 6 women). Ten patients had liver transplant (3 males and 7 females). The mean time of transplant was 32.8±24.1month (range: 4-72).

All patients were diagnosed for TTRVal30Met mutation by DNA analysis and were subjected to neurological evaluation to establish the clinical history and define disease stage. The staging system used was as follows: P0 – asymptomatic with a normal neurological examination; P1 – sensory disturbances in the lower extremities and mild autonomic disturbances;P2 – sensory and mild motor disturbances in the lower extremities, mild sensory disturbances in the hands and moderate autonomic disturbances; P3 –difficulty in walking (need for orthothesis or support),moderate sensory and motor disturbances in the hands and moderate autonomic disturbances;P4 – patients in a wheelchair or bed ridden, with severe autonomic neuropathy (Coutinho P et al 1980).The Ethical Committee of Hospital de Santo António approved this study and all subjects gave written informed consent before the LSG biopsy.

LSG biopsies were performed using local anesthesia (3% Lidocaine). The lip was everted, and a 0.5–10 mm longitudinal incision was made in the labial mucosa in front of the mandibular canine tooth and sutured with a reabsorbable monofilament. Two to four LSG were removed from each individual.

Collection of saliva samples

Saliva samples were collected from 68 patients (30 men, 38 women) and 9 control group subjects (2 men, 7 women). All patients were diagnosed for the TTRVal30Met mutation by DNA analysis and were subjected to neurological evaluation to establish the clinical history and define the clinical stage. The collection of stimulated whole saliva was performed under resting between 10:00 and 12:00 am. They did not ingest any type of foods, brushed the teeth, chew chewing gum or candies or ingest liquids.

Before starting the evaluation, the individuals swallowed all the saliva that they had in the mouth, at that moment.

During the five minutes of the measurement, the individuals remain seated bending over and with elbows rested on the thighs, in such a way that the head was tilted forward. At this position they stay relaxed, closing the eyes and breathing for the nose. Clinical data and environmental conditions were registered.

Saliva was collected in an appropriate tube with two compartments (Salivette TM, Sarstedt, Nümbrecht, Germany). The upper compartment contained a piece of cotton to filter the saliva that was recovered in the lower compartment after centrifugation at 3000 g for 10 minute. Saliva samples were then recovered and immediately frozen at -80º C.

Histopathological study of LSG

Labial salivary glands were fixed in a 10% formaldehyde solution and studied by optical microscopy. The histological specimens included in paraffin were stained with haematoxylin and eosin to evaluate the global aspect of the gland. The presence and distribution of amyloid deposits were evaluated by Congo red staining and observed under polarized light to confirm the characteristic apple green birefringence.

The global aspect of the LSG and the amount and distribution of the amyloid deposits was graded as follows: G0 – normal morphology without amyloid deposits; G1 – normal morphology of the gland and very discrete amyloid deposits in the interstitial connective tissue stroma or surrounding a few glandular acini; G2 – the global aspect of the gland is preserved with some discrete interstitial fibrosis and more evident amyloid deposits surrounding several glandular acini; G3 – the global aspect of the gland is altered with evident loss of the acinar tissue, pronounced interstitial fibrosis and very abundant amyloid deposits around all glandular tissue. Several slides were analyzed to confirm the results.

Immunohistochemistry

Five-micrometer thick tissue sections were deparaffinated in Histoclear1 (National Diagnostics, Atlanta, GA) and dehydrated in a descendent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/100% methanol, and sections were blocked with 4% foetal bovine serum and 1% bovine serum albumin in phosphate buffered solution (PBS). Primary antibodies used were as follows: rabbit polyclonal anti-Fas (Santa Cruz Biotechnology, Santa Cruz, CA,1:100), rabbit polyclonal anti-3-nitrotyrosine Chemicon International, Temecula, CA, 1:500), goat polyclonal anti-BiP (Santa Cruz Biotechnology, Santa Cruz, CA, 1:50) and finally rabbit polyclonal anti-TTR (Dako, Carpinteria, CA, 1:1000), which were diluted in blocking solution and incubated overnight at 48C. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma-Aldrich, St. Louis, MO), using 3-amino-9-ethyl carbazole (Sigma-Aldrich, St. Louis, MO), or diaminobenzidine as substrates. On parallel control sections, the primary antibody was replaced by blocking buffer and by non-immune immunoglobulins corresponding to the species used for primary antibodies, namely rabbit an goat; staining was absent under these conditions. Semiquantitative immunohistochemistry analyses were carried out with the Universal Imaging system (NIH, Bethesda, MD) which performs automated particle analysis in a measured area, that is, the area occupied by pixels corresponding to the immunohistochemical substrate's colour is counted and normalized relatively to the total area. Each slide was analyzed in 3 different selected areas. Results shown represent % occupied area ± SD.

ELISA

Saliva was collected from FAP patients and control volunteers centrifuged at 3,000 rpm for 10 min at 4ºC to remove insoluble material and immediately frozen at -80ºC until usage. Total Human Metalloproteinase-9 (MMP-9) was quantified by an enzyme-linked immunosorbent assay (ELISA) Kit from Quantikine (R&D Systems, Inc). Briefly, quality controls and samples are incubated in microplate wells pre-coated with polyclonal anti-human MMP-9 antibody. After 120 minutes incubation and washing, polyclonal anti-human MMP-9 antibody, conjugated with horseradish peroxidase (HRP) was added to

the wells and incubated for 120 minutes with captured MMP-9. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product was measured.
Absorbance was read with a Microtiter plate reader at 450 nm (Multiskan Ascent, Thermo Electron Corporation, USA). The absorbance is proportional to the concentration of MMP-9. A standard curve is obtained by plotting the absorbance vs. the concentration and the absorbance values for test samples were used to determine the concentration from the standard curve.

Statistical analysis

All data examined were expressed as mean ± S.D. Comparison between groups was made using the t-Student and ANOVA tests. Spearman's correlation coefficients were used to establish the association between staging and amyloid deposition. Pearson's correlation was used to establish the association between MMP-9 and age. A p value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 15.0 for Windows (SPSS, Chicago IL.).

Results

Glandular MMP-9

MMP-9 and neurological staging

Neurological evaluation was performed as established by Coutinho and collaborators (Coutinho P et al 1980). Accordingly, all 10 asymptomatic carriers (6 males and 4 females) were classified as P0; 10 patients (3 males and 7 females) had only mild sensory and autonomic neuropathy and were classified as P1; 6 patients (3 males and 3 females) had moderate sensory and autonomic neuropathy with mild motor problems and were classified as stage 2; 6 patients (2 males and 4 females) were in stage 3; no stage 4 patients were studied.

Clinical characteristics of the individuals studied are summarized In Table I and continuous variables are given as means±SD.

	Male	Female	Age Range	Mean Age (SD)
	n	n		
Normal	4	6	18-51	36.1 (7.9)
FAP0	6	4	27-53	33,90 (8.5)
FAP1	3	7	29-45	35,00 (5.1)
FAP2	3	3	33-58	46,33 (10.4)
FAP3	2	4	34-65	45,67 (10.4)
LT	3	7	24-52	40.0 (8.4)
Total	21	31	24-65	39.7 (9.4)

Table I. Clinical characteristics of the glandular MMP-9 sample

The mean level of MMP-9 for the control group±SD was 158.9±180.8. Mean value of male subjects was 248.6±244.5 and the mean level of female subjects was 98.5±110.4.

In FAP0 the mean level of MMP-9 ±SD was 439.2±702.1.. Mean value of male subjects was 404.0±775.4 and the mean level of female subjects was 491.9±686.1.

In FAP1 the mean level of MMP-9 ±SD was 490.1±651. Mean value of male subjects was 158.7± 182.9 and the mean level of female subjects was 632.1 ±740.0.

In FAP2 the mean level of MMP-9 ±SD was 1223.±1608.2. Mean value of male subjects was 2379.9± 1565.8 and the mean level of female subjects was 66.9±34.0.

In FAP3 patients the mean level of MMP-9 ±SD was 3723.9±2510.4. Mean value of male subjects was 4904.9± 2980.5 and the mean level of female subjects was 3133.4±2479.4.

In liver transplant patients the mean level of MMP-9 ±SD was 230.2±413.0.Mean value of male subjects was 547.6± 738.4 and the mean level of female subjects was 94.2±46.8. Distribution of MMP-9 glandular levels by the staging disease is summarized in Table 2.

MMP-9	Male			Female			Age
%occupied area	n	M (SD)		n	M (SD)		Range
Normal	4	248,65	(244,50)	6	98,54	(110,42)	18 - 51
FAP0	6	404,01	(775,36)	4	491,87	(686,09)	27 - 53
FAP1	3	158,73	(182,85)	7	632,11	(739,98)	29 - 45
FAP2	3	2.379,98	(1.565,83)	3	66,88	(33,98)	33 - 58
FAP3	2	4.904,95	(2.980,53)	4	3.133,42	(2.479,42)	34 - 65
LT	3	547,63	(738,36)	7	94,21	(46,82)	24 - 52

Table 2.Distribution of MMP-9 glandular levels by the staging disease.

ANOVA show statistically significant differences in MMP-9 LSG levels in patients at different disease stages were compared with control individuals (F=10.5; p=0.05). When we used the Post-hoc tests, statistically significant differences were found only between stages P2 and P3.The results obtained for LSG MMP-9 levels are represented against the disease Stage (Figure 1)

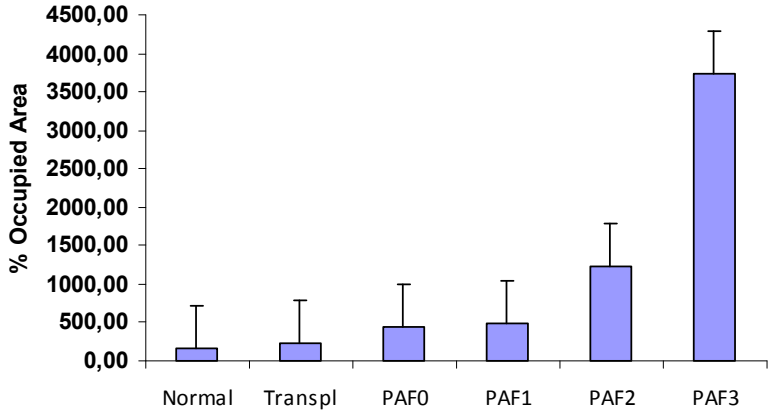

Figure 1. Histograms of quantification of TTR images. Quantification of TTR images are represented as percentage of occupied area±SD (*, p≤0.05)

MMP-9 and amyloid deposition

Frequencies for amyloid distribution were the following: 25 patients in G0; 1 patient in G1; 3 in G2 and 13 in G3. For statistical purpose not was considered stage G1 and G2 due to the small number of subjects. We found higher levels of MMP-9 in males than females (602.2 and 4631.8 respectively), but these differences weren't statistically significant (G0: t=0.67; *p*= 0.492; G; *t*=1.94; *p*=0.07), neither between different groups (*F*= 2.36; *p*= 0.08). In relation to amyloid deposition there was no statistically significant differences between groups (p>0.05). However when using the Pearson correlation we found a positive correlation between levels of glandular MMP-9 and deposition of amyloid substance (r=0.39; p=0.01).

MMP-9	**Male**		**Female**	
	n	**M (SD)**	**n**	**M (SD)**
G0	13	602,22 (1.150,55)	12	348,17 (529,79)
G1	1	882,63 .	0	
G2	2	1.434,86 (1.926,91)	1	28,58
G3	2	4.631,75 (3.366,90)	11	1.410,10 (1.997,44)

Table 3. Frequencies of amyloid distribution

Figure 2 shows representative images of immunohistochemistry analysis obtained from patients at different stages of the disease. MMP-9 levels were determined as the percentage of area occupied by MMP-9 staining.

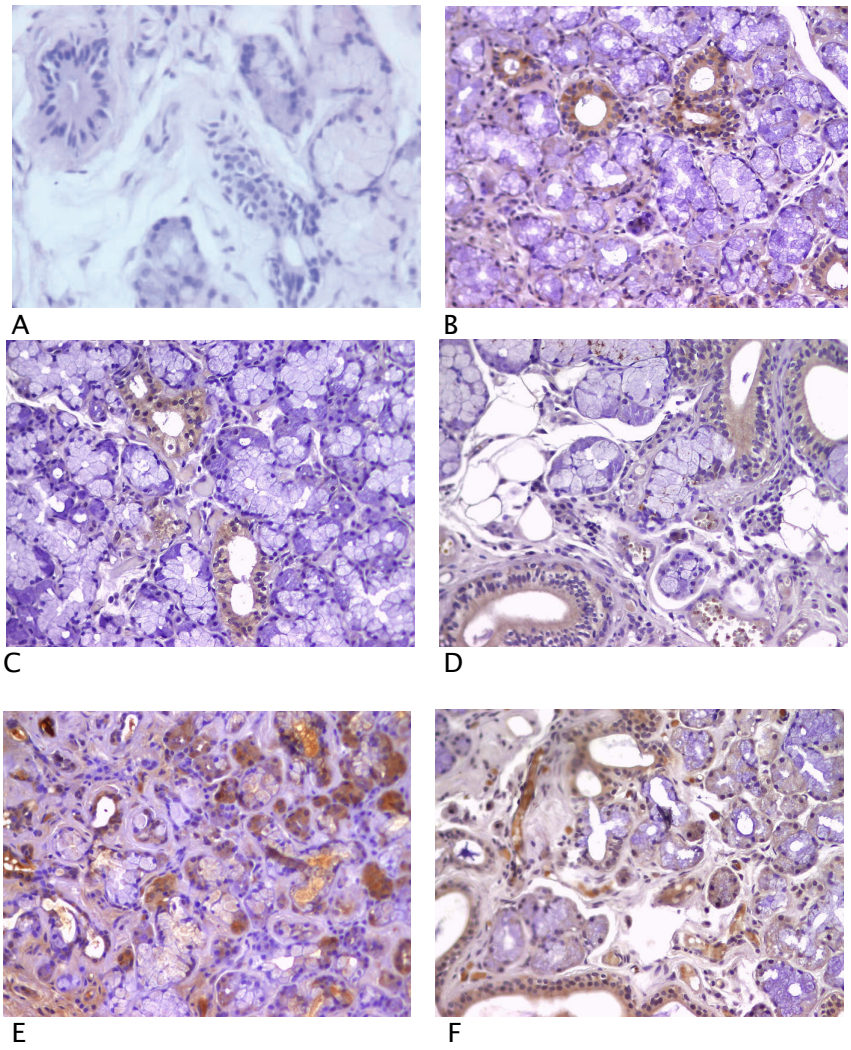

Figure 2. Immunohistochemistry of MMP-9 in salivary glands from individuals at different stages of disease progression. A. controls (healthy individuals); B. FAP transplanted; C. FAP 0; D. FAP 1; E. FAP 2: F. FAP 3. 20 x magnifications. Quantification of TTR staining is represented as percentage of occupied area±SD ($p \leq 0.05$)

Salivary MMP-9

Salivary MMP-9 and disease stage

The main clinical features of patients and controls as well the results are as follow: 21 asymptomatic carriers were classified as P0 (11 male and 10 females); 38 patients (17 males and 21 females) were classified as P1; 9 subjects (2 males and 7 females) were the control group. There were not sufficient samples of P2 and P3 to be measured.

MMP-9 (µg/ml)	Male n	Female n	Age Range	Mean Age (SD)
Normal	2	7	27-70	50,45 (13,9)
FAP0	11	10	21-46	30,40 (5.7)
FAP1	17	21	25-70	36,58 (10,5)
Total	30	38	23-70	37,51 (12,3)

Table 3. Clinical characteristics of the salivary sample

The mean level of the asymptomatic subject's ±SD was 39.8µg/ml± 20.0 µg/ml Mean value of male subjects was 47.8 µg/ml±9.6 and the mean level of female subjects was 37.5µg/ml ±22.1 µg/ml.

In FAP 0 the mean level of MMP-9 ±SD was 31.6µg/ml±13.6µg/ml.Mean value of male subjects was 34.4 µg/ml± 15.6 and the mean level of female subjects was 28.4µg/ml ±11.0µg/ml.

In FAP1 the mean level of MMP-9 ±SD was 30.2µg/ml±14.4µg/ml.Mean value of male subjects was 29.7 µg/ml± 13.0 and the mean level of female subjects was 30.6µg/ml ±15.7µg/ml However no statistical differences were found (F= 1.524; p=0.226).

Table 4 shows the salivary MMP-9 distribution between male and female subjects. We found higher values in males in normal and P0 (47.8µg/ml and 34.2µg/ml, respectively). However these differences weren't statistically significant (t= 0.615; p=0.558 in normal and t=1.006; p=0.327). Although there are higher salivary MMP-9 levels in female P1 the difference was not statistically significant (t= –0.190; p= 0.850).

MMP-9	Male			Female		
(µg/ml)	n	M (SD)		n	M (SD)	
Normal	2	47,80	(9,62)	7	37,54	(22,13)
FAP0	11	34,42	(15,64)	10	28,42	(11,00)
FAP1	17	29,66	(12,95)	21	30,56	(15,73)

Table 4. Salivary MMP-9 levels distribution by disease staging.

Salivary MMP-9 and amyloid deposition

Frequencies for salivary amyloid distribution were the following: 32 patients (12 male, 18 females) in G0; 12 patients (7 male, 5 females) in G1; 2

patients (1 male, 1 female) in G2 and 22 (8 male, 14 females) in G3. There are no statistically significant differences between salivary MMP-9 and amyloid deposition (Table 5).

MMP-9	Male			Female		
(µg/ml)	n	M (SD)		n	M (SD)	
G0	14	35,09	(15,67)	18	32,22	(15,60)
G1	7	36,24	(10,26)	5	33,36	(9,53)
G2	1	33,90		1	16,20	
G3	8	24,94	(14,12)	14	30,41	1(8,74)

Table 5. Salivary MMP-9 and amyloid deposition

Discussion

In the oral cavity, the main source of type IV colagenases has been suggested to be polymorphonuclear leukocytes (Hibbs MS et al 1985), macrophages (Mainardi CL et al 1984), and epithelial cells (Salo T et al 1991). Gingival keratinocytes produced mainly MMP-9, while gingival and granulation tissue fibroblasts expressed MMP-2. Glandular tissue contained mainly MMP-9, and mRNA for MMP-9 was also found in acinar epithelial cells (Mäkelä M et al 1994)

Important changes take place in the structure and function of the parenchyma and extracellular matrix (ECM) of salivary glands in patients with Sjögren Syndrome, resulting in glandular atrophy in patients with severe disease (Goicovich et al 2003).

In those patients, MMPs 2, 3 and 9 were detected in tissues sections of labial salivary glands using specific antibodies. MMPs were localized in ductal cells and in the basal region of mucous acinar cells, but were found preferentially in cells of the seromucous demilunes. Immunoreactivity for MMP-2 in control subjects was similar to that found in Sjögren patients. In contrast, while MMP-9 immunoreactivity in control subjects resembled that of MMP-2, a marked increase in MMP-9 immunoreactivity was observed in Sjögren patients (Pérez P et al 2000).

Although the pathogenic mechanisms are different in Sjögren's syndrome, in FAP patients the structure of labial salivary glands are in general altered with large amounts of amyloid deposition in most severe cases, in addition to fibrosis leading to salivary gland atrophy.

In a previous study with FAP patients we demonstrate that matrix metalloproteinase-9 (MMP-9) which exists as a complex with neutrophil gelatinase-associated lipocalin (NGAL), was increased in FAP and in vitro degraded TTR aggregates and fibrils. Byglican, NGAL and MMP-9 are transcriptionally up-regulated by NF-kB, a transcription factor that is activated in FAP nerves and salivary glands (Sousa MM et al 2004).

The present study confirms that MMP-9 is expressed in acinar and ductal cells of labial salivary glands and shows that MMP-9 levels vary in different stages of the disease and are statistically significant.

When comparing glandular MMP-9 distribution by staging there are significantly differences between all the groups studied (f=10.5; p=0.000).The

highest levels of glandular MMP-9 intensity were detected in patients having a more disabling disease. Our data are similar to those reported by Pérez et al in 2000.

Transplant patients had a lower level than FAP 0, FAP 1; FAP 2 and FAP 3, probably due to the immunosuppression.

Salivary MMP-9 levels were higher in males in normal and FAP0, but the differences weren't statistically significant. Although there are higher salivary MMP-9 levels in FAP1 the difference was not statistically significant.

The presence of MMP-9 in patients with primary SS has been investigated by Konttinen et al in 1998, using saliva samples. MMPs are enzymes acting upon the ECM components, and they are constitutively secreted exclusively from the basal plasma membrane of acinar and ductal cells. Thus, the question arises of how MMP-9 could be expected to be present in saliva. Some investigators postulated that both the basal and apical surfaces of these cells are damaged (Goicovich et al 2003). Others have suggested that a loss of acinar epithelium polarity occurs, which may lead to an alteration of MMP secretion patterns (Wilson CL et al 1997). As a consequence of the loss in acinar cell organization, MMPs in SS patients may be targeted incorrectly to the apical membrane, and are therefore present in saliva.

In present study, salivary MMP-9 levels were higher in males in normal and FAP0, but the differences weren't statistically significant. There are higher salivary MMP-9 levels in FAP1 but the difference was not statistically significant.

Although we have not measured the levels of salivary MMP-9 in stages P2 and P3 due to insufficient number of samples, higher levels of salivary MMP-9 were found in FAPG0 and FAPG1, so it is expected that in the advanced stages of disease there are higher levels of MMP-9 in saliva.

The great variability between glandular changes, amyloid deposition and xerostomia and the existence of other local factors that can interfere with metalloproteinases could explain our data. The great variability in sample composition mainly the existence of few patients in each stage of the disease may explain the wide variation in results. Others studies are needed to confirm our data, involving an equal number of patients in various stages of the disease so that the sample studied is more homogeneous.

V.B. ZAG levels in FAP labial salivary glands and saliva in TTRVal30Met carriers.

Contributors to this paper:

Dras. Tania Ribeiro[1] and Diana Martins[1] in Immunocytochemistry and ELISA processing samples.
Dr. Antonio Guimarães[2] in histological interpretation, analysis and discussion of the results.

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Abstract

Background: Familial amyloidotic polyneuropathy (FAP) is a disorder characterized by extracellular deposition of fibrillar transthyretin (TTR) amyloid with a special involvement of the peripheral nerves. Zinc-α2-glycoprotein (ZAG) is expressed in human saliva and is present in the luminal epithelial cells in the intercalated portions of some ducts, as well in excretory ducts. Recently, the gene expression of ZAG in normal epidermal and buccal epithelia was reported. Due to the presence of arginine-glycine-aspartase (RGD) (residues 231-233) in α3 domain, it may attain the properties of cell adhesion between cells and extracellular matrices. Integrin-mediated cell adhesion requires divalent Mg^{2+} cations, and ZAG also shows adhesion in the presence of Mg^{2+} and Mn^{2+}.

Objectives: To assess the ZAG levels in labial salivary glands (LSG) and in saliva of PAP patients at different stages of the disease, show the histological localization of ZAG in salivary gland tissues and correlate these parameters with the severity of the disease.

Methods: Labial salivary glands were obtained from 52 patients (21 males, 31 females) and 10 control subjects (4 males, 6 females). Saliva sample were collected from 68 patients (30 males, 38 females) and 9 control subjects (2 males, 7 females). All patients were diagnosed for TTRVal30Met mutation by DNA analysis and submitted to a neurological observation to determine the neurological stage of the disease. Ten patients (3 males, 7 females) had liver transplant. The mean time of transplant was 32.8 months (±24.1 SD).

Results: When we compared the levels of ZAG gland at different stages of the disease did not show statisticaly significant differences. A positive correlation was found between amyloid deposition and salivary ZAG and for salivary ZAG and disease stage.

Conclusions: We show some differences in ZAG levels in labial salivary glands and saliva between FAP patients and the control group. We must however emphasize that with regard to salivary ZAG we studied only asymptomatic carriers and stage P1 so these data should be interpreted with caution.

Introduction

Familial amyloid polyneuropathy (FAP) is a neurodegenerative autosomal dominant disorder characterized by the systemic extracellular deposition of mutated transthyretin amyloid fibrils in several tissues, particularly in the peripheral nervous system (Andrade C 1952). The most common point of mutation in TTR is a substitution of valine for methionine at position 30 of the polypeptide chain, which promotes amyloidogenesis (Saraiva MJ et al 1983).

In amyloid related-disorders, amyloid deposits are made of two types of components: the disease-specific peptide/protein and amyloid-associated molecules that include serum amyloid P component (Pepys MB et al 1994), apolipoprotein E (Gallo G et al 1994) and extracellular-matrix related components such as proteoglycans (Lyon A et al 1991).

Zinc α2-glycoprotein (ZAG) is a 40-kDa single chain polypeptide ZAG that was first reported in human serum and subsequently purified (Bürgi W, Schmid K 1961) and is secreted in various body fluids (Tada T et al 1991). ZAG is known to stimulate lipolysis in murine epididymal adipocytes trough stimulation of adenylate cyclase in a GTP-dependent process via binding trough β_3-adrenoreceptor (Russell ST and Tisdale MJ 2005).

It is involved preferentially in depletion of fatty acids from adipose tissues, subsequently named as lipid-mobilizing factor (Bao Y et al 2005). This factor, which is highly expressed in cancer cachexia, is characterized by the extensive reduction of fat in the human body (Bing C et al 2004).

The protein assay (Diez-Itza et al 1993) and mRNA expression in the mammary tumor (Freije JP et al 1991) have shown that there is a relation between ZAG levels and histologic grade of the breast cancer tumors. Moreover, many studies suggested that ZAG is also a potential serum marker of prostate cancer that may be elevated early in tumor growth (Frenette G et al 1987).

The crystal structure of ZAG consists of a large groove analogous to class I MHC peptide-binding grooves and might be able to bind with different peptides, antigens and ligands (Sánchez LM et al 1999).

The presence of ZAG in human seminal fluid was reported in 6-fold molar excess compared with human serum (Ohkubo I et al 1990), which suggested that ZAG is a key element for fertilization without any experimental evidences.

Serum ZAG is synthesized by the genes of the liver. Among proteins expressed in human saliva, ZAG was determined by proteomic analysis and mass finger printing.

ZAG has been detected in saliva samples from Portuguese FAP patients using proteomic analysis by Macedo B et al 2007.

Gene expression of ZAG in normal human epidermal and buccal epithelia was reported (Brysk MM et al 1997). ZAG is also produced by adipocytes, where the mRNA of ZAG was detected by reverse transcription-PCR in the mouse white adipose tissue and in the interscapular brown fat. Finally, ZAG is synthesized by epithelial cells of prostate gland and liver, secreted into various body fluids such as serum, semen (Bürgi W et al 1989), sweat, saliva, cerebrospinal fluid, milk (Bundred NJ et al 1987),urine (Jain S et al 2005), and amniotic fluid (Ding M et al 1990).

The concentration of ZAG has been reported to dramatically increase in carcinomas. Therefore, it is also considered as a good biomarker for prostate (Henshall SM et al 2006), breast (Sánchez LM et al 1999), oral (Brysk MM et al 1997), and epidermal carcinomas (Lei G et al 1997).

The gene expression of ZAG from histopathologically graded oral squamous cell carcinomas was compared. It was observed that ZAG levels are higher in well-differentiated tumors than those in the poorly differentiated tumors. These findings led to the conclusion that ZAG can also be considered as a marker of the oral epithelial maturation (Brysk MM 1997).

In salivary glands, the basal lamina in acini is connected to the cytoskeleton of acinar cells via integrins in the basal plasma membrane (Ashkenas J et al 1996; Yeaman C et al 1999). Due to the presence of Arg-Gly-Asp (residues 231-233) in α3 domain, it may attain the properties of cell adhesion between cells and extracellular matrices (Lei G et al 1999). Based on the RGD sequence, Takagaki et al 1994, showed the attachment and spreading of various cells on ZAG-coated plates, and they found lower cell spreading as compared with fibronectin, laminin, fibrinogen, and vitronectin. Cell spreading for serum and seminal ZAG was the same despite many posttranslational differences. Furthermore, they used RGD-containing synthetic peptides and observed appreciable inhibition in cell spreading and suggested that RGD region of ZAG plays an important role in cell spreading. Integrin mediated cell adhesion requires divalent Mg^{2+} cations, and ZAG also shows adhesion only in the presence of Mg^{2+} and Mn^{2+}.

The aim of this study was to investigate if the levels of ZAG in labial salivary glands and saliva could explain the changes in salivary gland histology as a possible co-factor related to amyloid deposition, and whether could serve as a biomarker to distinguish precocious staging of the disease and correlate with disease progression.

Material and methods

Labial salivary glands biopsies

Labial salivary glands (LSG) were obtained from 52 individuals (21 men, 31 women) and 10 control subjects (4 men, 6 women). Ten patients had liver transplant (3 males and 7 females). The mean time of transplant was 32.8±24.1 month (range: 4-72).

All patients were diagnosed for TTRVal30Met mutation by DNA analysis and were subjected to neurological evaluation to establish the clinical history and define the clinical stage (Coutinho P et al 1990). The Ethical Committee of Hospital de Santo António approved this study and all subjects gave written informed consent before the LSG biopsy. LSG biopsies were performed using local anesthesia (3% Lidocaine). The lip was everted, and a 0.5–10 mm longitudinal incision was made in the labial mucosa in front of the mandibular canine tooth and sutured with a reabsorbable monofilament. Two to four LSG were removed from each individual.

Collection of saliva samples

Saliva samples were collected from 68 patients (30 men, 38 women) and 9 control group subjects (2 men, 7 women). The collection of stimulated whole saliva was performed under resting between 10:00 and 12:00 am. They did not ingest any type of foods, brushed the teeth, had chewing gum or candies or ingest liquids.

Before starting the evaluation, the individuals swallowed all the saliva that they had in the mouth, at that moment.

During the five minutes of the measurement, the individuals remain seated bending over and with elbows rested on the thighs, in such a way that the head was tilted forward. At this position they stay relaxed, closing the eyes and

breathing for the nose. Clinical data and environmental conditions were registered.

Saliva was collected in an appropriate tube with two compartments (Salivette TM, Sarstedt, Nümbrecht, Germany). The upper compartment contained a piece of cotton to filter the saliva that was recovered in the lower compartment after centrifugation at 3000 g for 10 minutes. Saliva samples were then recovered and immediately frozen at -80º C.

Histopathological study of LSG

Labial salivary glands were fixed in a 10% formaldehyde solution and studied by optical microscopy. The histological specimens included in paraffin were stained with haematoxylin and eosin to evaluate the global aspect of the gland. The presence and distribution of amyloid deposits were evaluated by Congo red staining and observed in polarized light to confirm the characteristic apple green birefringence.

The global aspect of the LSG and the amount and distribution of the amyloid deposits was graded as follows: G0 – normal morphology without amyloid deposits; G1 – normal morphology of the gland and very discrete amyloid deposits in the interstitial connective tissue stroma or surrounding a few glandular acini; G2 – the global aspect of the gland is preserved with some discrete interstitial fibrosis and more evident amyloid deposits surrounding several glandular acini; G3 – the global aspect of the gland is altered with evident loss of the acinar tissue, pronounced interstitial fibrosis and very abundant amyloid deposits around all glandular tissue. Several slides were analyzed to confirm the results.

Immunohistochemistry

Five-micrometer thick tissue sections were deparaffinated in Histoclear1 (National Diagnostics, Atlanta, GA) and dehydrated in a descendent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/100% methanol, and sections were blocked with 4% fetal bovine serum and 1% bovine serum albumin in phosphate buffered solution (PBS). Primary antibodies used were as follows: ZAG antibody were diluted in blocking solution and incubated overnight at 48C. Antigen visualization was performed with the biotin-extravidin-

peroxidase kit (Sigma-Aldrich, St. Louis, MO), using 3-amino-9-ethyl carbazole (Sigma-Aldrich, St. Louis, MO), or diaminobenzidine as substrates. On parallel control sections, the primary antibody was replaced by blocking buffer and by non-immune immunoglobulins corresponding to the species used for primary antibodies, namely rabbit an goat; staining was absent under this conditions. Semiquantitative immunohistochemistry analyses were carried out with the Universal Imaging System (NIH, Bethesda, MD), which performs automated particle analysis in a measured area, which is the area occupied by pixels corresponding to the immunohistochemical substrate's color and it is counted and normalized relatively to the total area. Each slide was analyzed in 3 different selected areas. Results shown represent % occupied area ± SD.

ELISA

Saliva was collected from FAP patients and control volunteers centrifuged at 3,000 rpm for 10 min at 4ºC to remove insoluble material and immediately frozen at -80ºC until usage. Total ZAG was quantified by an enzyme-linked immunosorbent assay (ELISA) Kit from Quantikine (R&D Systems, Inc). Briefly, quality controls and samples are incubated in microplate wells pre-coated with polyclonal anti-human ZAG antibody. After 120 minutes incubation and washing, polyclonal anti-human ZAG antibody, conjugated with horseradish peroxidase (HRP) was added to the wells and incubated for 120 minutes with captured ZAG. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product was measured. Absorbance was read with a Microtiter plate reader at 450 nm (Multiskan Ascent, Thermo Electron Corporation, USA). The absorbance is proportional to the concentration of ZAG. A standard curve is obtained by plotting the absorbance vs. the concentration and the absorbance values for test samples were used to determine the concentration from the standard curve.

Statistical analysis

All the data examined was expressed as mean ± S.D. Comparison between groups was made using the t-Student and ANOVA tests. A p value of less than 0.05 was considered statistically significant. Spearman's correlation coefficients were used to establish the association between staging and amyloid deposition. Pearson's correlation was used to establish the association between ZAG and age, and stage. Statistical analysis was performed using SPSS version 15.0 for Windows (SPSS, Chicago, IL.).

Results

Glandular ZAG

ZAG and neurological staging

Neurological evaluation was performed as established by Coutinho and collaborators (Coutinho P et al 1980). Accordingly, all 10 asymptomatic carriers (6 males and 4 females) were classified as P0; 10 patients (3 males and 7 females) had only mild sensory and autonomic neuropathy and were classified as P1; 6 patients (3 males and 3 females) had moderate sensory and autonomic neuropathy with mild motor problems and were classified as stage 2; 6 patients (2 males and 4 females were in stage 3; no stage 4 patients were studied. The body mass index (BMI) for FAP patients was 24.8 and for the control group was 25.7.Ten patients had liver transplant (3 males and 7 females). The mean time of transplant was 32.8±24.1 month (range: 4-72)

Clinical characteristic of the individuals studied are summarized in Table 1 and continuous variables are given as means±SD.

	Male	Female	Age	Mean Age
	n	n	Range	(SD)
Normal	4	6	18-51	36.1 (7.9)
FAP0	6	4	27-53	33,90 (8.5)
FAP1	3	7	29-45	35,00 (5.1)
FAP2	3	3	33-58	46,33 (10.4)
FAP3	2	4	34-65	45,67 (10.4)
LT	3	7	24-52	40.0 (8.4)
Total	21	31	24-65	39.7 (9.4)

Table 1. Clinical characteristics of the glandular ZAG sample

The mean level of ZAG for the control group±SD was 17887.1± 7537.2. Mean value of male subjects was 19050.8 ±9689.1 and the mean level of female subjects was 16723.5 ±5921.4.

In FAP0 the mean level of ZAG ±SD was 17571.0±9089.4. Mean value of male subjects was 13932.6 ± 4036.8 and the mean level of female subjects was 22119.0±12204.2.

In FAP1 the mean level of ZAG ±SD was 12780.0±12774.0. Mean value of male subjects was 20961.0± 8405.8 and the mean level of female subjects was 9273.9±13168.3.

In FAP2 the mean level of ZAG ±SD was 22770.5±26583.9. Mean value of male subjects was 9517.0± 6105.2 and the mean level of female subjects was 36024.0 ±34667.3.

In FAP3 the mean level of ZAG ±SD was 36637.09±19937.7. Mean value of male subjects was 52090.5 ± 20969.3 and the mean level of female subjects was 28910.3±16647.4.

In liver transplant patients the mean level of ZAG ±SD was 13129.5±7757.3. Mean value of male subjects was 16539.0 ± 13710.9 and the mean level of female subjects was 11668.3±4392.9.

ANOVA was statistically significant (F=2.7; p=0.032) but when comparing ZAG levels in different staging using the Post-hoc test, these differences were not confirmed.

Distribution of ZAG glandular levels by the staging disease are summarized in Table2.

ZAG	Male			Female			Age
% occupied area	n	M (SD)		n	M (SD)		Range
Normal	4	19050,75	(9689,15)	4	16723,50	(5921,44)	18 - 51
FAP0	5	13932,60	(4036,83)	4	22119,00	(12204,18)	27 - 53
FAP1	3	20961,00	(8405,83)	7	9273,86	(13168,33)	29 - 45
FAP2	3	9517,00	(6105,20)	3	36024,00	(34677,30)	33 - 58
FAP3	2	52090,50	(20969,25)	4	28910,25	(16647,42)	34 - 65
LT	3	16539,00	(13710,92)	7	11668,29	(4392,89)	24 - 52

Table 2. Distribution of ZAG glandular levels by disease staging

Figure 1 shows representative images of immunohistochemistry analysis obtained from patients at different stages of the disease. ZAG levels were determined as the percentage of area occupied by ZAG staining. The results obtained for ZAG levels are represented against the disease stage of the individuals analyzed in Figure 1.

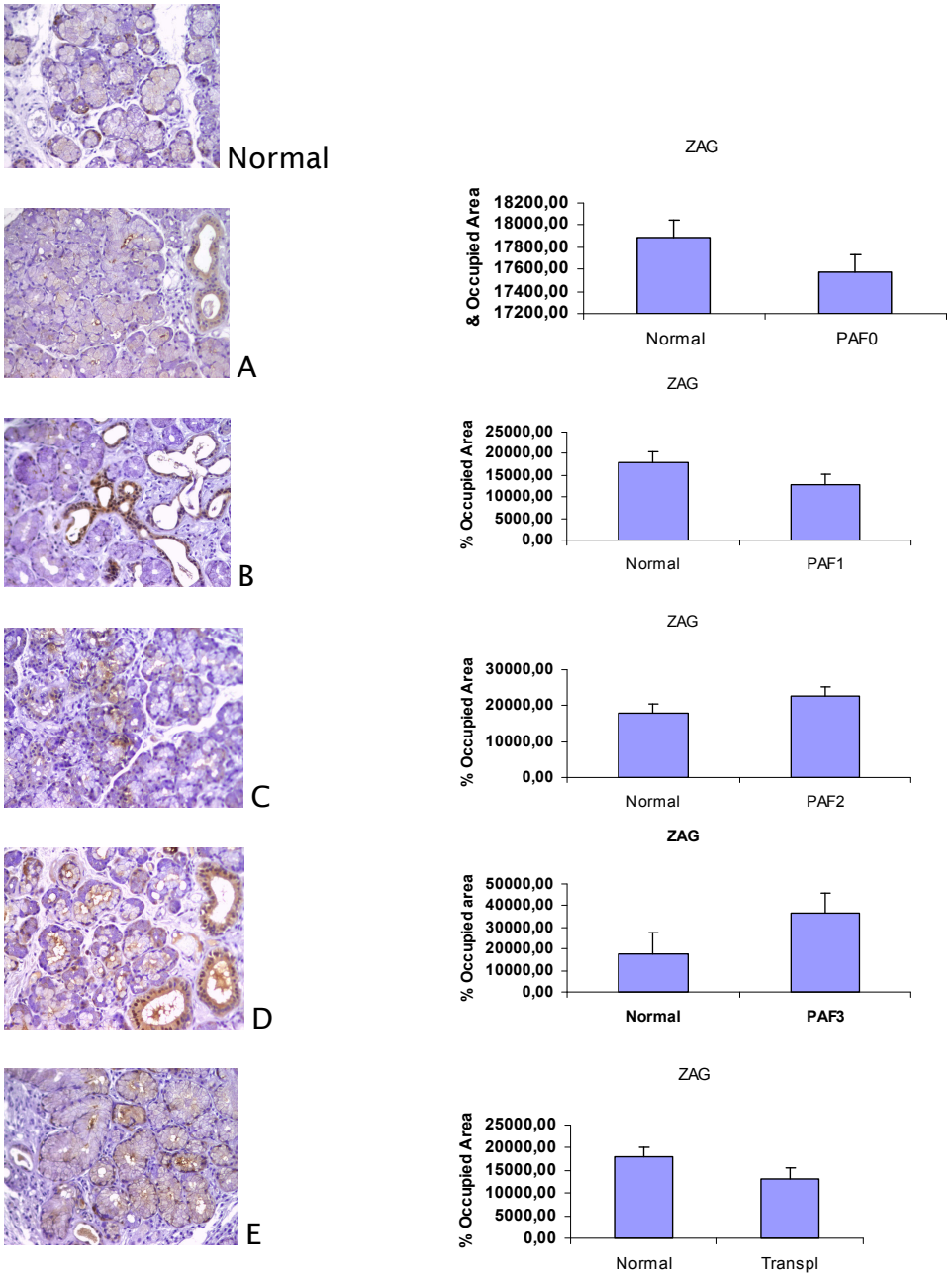


Figure 1. Immunohistochemistry of ZAG in labial salivary glands from individuals at different stages of disease. On the left top a normal histology from LSG; A. FAP 0; B. FAP 1; C FAP 2; D FAP 3; E. FAP TX. 20 x magnifications. Quantification of TTR staining is represented as percentage of occupied area ±SD ($p \leq 0.05$)

ZAG and amyloid deposition

From the 52 patients who underwent LSG, 25 patients (13 male and 12 female) showed that the gland morphology was preserved and there was no amyloid deposition (G0 staging).In one male patient the global gland morphology was normal but we could observe very sparse amyloid deposits in the connective tissue (G1 staging).Three patients (2 male and 1 female) presented mild interstitial fibrosis and more pronounced amyloid deposition (G2 staging) and in 13 patients (2 male and 11 female) the global glandular aspect was severely changed with profuse amounts of amyloid (G3 staging) This data are summarized in Table 3.

We found higher levels of ZAG in females G0 e G3 (16489.2µg/ml and 23976.8µg/ml, respectively). However, these differences were not statistically significant between different groups (F= 1.82; p= 0.161).

ZAG	Male		Female	
	n	M (SD)	n	M (SD)
G0	12	16471,50 (6664,66)	10	16489,20 (10323,30)
G1	1	7845,00	0	
G2	2	47146,50 (27961,12)	1	15363,00
G3	2	20842,50 (23222,09)	11	23976,82 (23463,07)

Table 3. ZAG distribution by amyloid deposition

Salivary ZAG

Salivary ZAG and disease stage

The main clinical features of patients and controls, as well as the results are as follows: 15 asymptomatic carriers were classified as P0 (7 male and 8 females); 33 patients (17 males and 16 females) were classified as P1; 11 subjects (2 males and 9 females) were the control group (Table 4).

ZAG	Male			Female		
(µg/ml)	n	M (SD)		n	M (SD)	
Normal	2	44,65	(13,36)	9	35,02	(11,22)
FAP0	7	39,67	(12,58)	8	35,85	(16,32)
FAP1	17	42,80	(13,15)	16	35,18	(12,57)

Table 4. Salivary ZAG and disease stage.

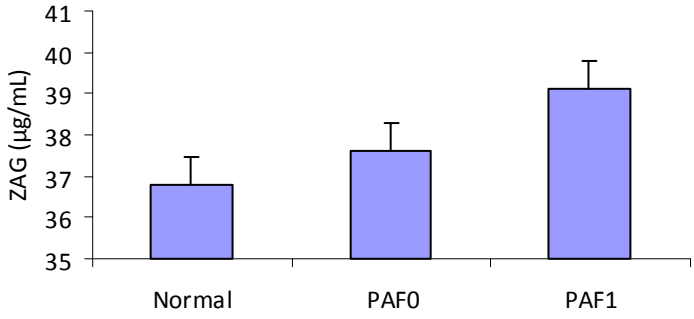
In control group the mean level of ZAG±SD was 36.8µg/ml± 11.6µg/ml Mean value of male subjects was 44.7µg/ml±13.4µg/ml and the mean level of female subjects was 35.0 µg/ ml ±11.2 µg/ml.

In FAP 0 the mean level of ZAG ±SD was 37.6µg/ml±14.3µg/ml.Mean value of male subjects was 39.7µg/ml± 12.6 and the mean level of female subjects was 35.9µg/ml ±16.3µg/ml.

In FAP 1 the mean level of ZAG ±SD was 39.1µg/ml±13.2µg/ml.Mean value of male subjects was 42.8 µg/ ml± 13.1 and the mean level of female subjects was 35.2µg/ml ±12.6µg/ml

Females had a lower ZAG level, being this difference statistical significant (t= 2.04; p=0.046).

Figure 2 shows a histogram of salivary ZAG distribution by disease staging.



Salivary ZAG and amyloid deposition

Distribution of salivary ZAG by amyloid deposition is summarized in Table 5. There were not statistically significant differences between male and females. For male and female FAP patients, Spearman correlation was positive for amyloid and salivary ZAG and for salivary ZAG and disease staging. In contrast to men, there was a positive correlation between amyloid and age, whereas for women that correlation was negative.

ZAG	**Male**			**Female**		
(µg/ml)	**n**	**M (SD)**		**n**	**M (SD)**	
g0	11	37,74	(13,46)	18	33,66	(13,81)
g1	6	38,02	(10,18)	3	37,00	(16,36)
g2	2	59,80	(1,41)	1	21,80	
g3	7	47,40	(9,07)	11	38,75	(10,47)

Table 5. Distribution of salivary ZAG by amyloid deposition

Discussion

Sánchez L.M et al 1999 identified ZAG as a 41-kDa soluble protein, as a fat-depleting factor, which is related to MHC class I molecules. Although its biological functions are incompletely understood, it seems to be a novel adipokine that may be involved in the local regulation of adipose tissue function (Bao Y et al 2005). Immunochemical localization of ZAG in FAP labial salivary glands is similar to those seen in normal salivary glands (Tada T et al 1991).

ZAG plays an important role in cell spreading (Takagaki M et al 1994). Integrin mediated cell adhesion requires divalent Mg^{2+} cations and ZAG also shows adhesion only in the presence of Mg^{2+} and Mn^{2+}.These facts may contribute to the histological changes seen in FAP labial salivary.

When we compared glandular ZAG and disease stage, ZAG was expressed in normal salivary glands to the same level in different disease staging except for more advanced staging disease, where ZAG levels are higher.

Concerning the glandular ZAG and amyloid distribution, we found higher levels of ZAG in females but these differences were not statistically significant. According to the study by Dubois et al in 2010 in patients with breast cancer these differences may be related to metabolic pathways involving estrogens or adipokine. However, it must be emphasized that for statistical purposes we did not consider stages G1 and G2, due to the small number of subjects.

We studied the salivary ZAG in the control group and the asymptomatic FAP and stage 1. We found a positive correlation between salivary ZAG, amyloid deposition and disease stage in males and females. We found lower ZAG levels in females and the difference was statistically significant. In the present study we observed a negative correlation for females, between disease staging and age.

Salivary ZAG and amyloid distribution was studied only in G0 and G3 FAP patients. We found higher levels of salivary ZAG in males. However, these differences were not statistically significant between males and females, neither between different groups. In patients in chronic hemodialysis elevated ZAG serum levels were found compared with control, because renal filtration appears to be an important route of ZAG elimination (Philipp A et al 2010). Higher levels of salivary ZAG in this study in more advances stage of the disease probably can be explained by renal impairment associated with amyloid deposition (Lobato L 2004).

ZAG has been related with cachexia in cancer patients. It is important to understand the role of this adipokine in FAP cachexia presented in later stages of the disease. In this present study we did not involve FAP patients with more advanced disease. Our FAP patients have a BMI normal (24.8), so that we can establish any correlation with ZAG and cachexia.

In FAP labial salivary glands the different data of this study can be related with the glandular histological changes, namely the interaction of amyloid distribution and ZAG localization. It should, however, be noted that the fact that it was not possible to study all stages of the disease affects our results. Further studies with a great number of patients must be conducted in order to establish the pathophysiological mechanism of ZAG involved in FAP salivary glands and its consequences in oral health.

VI: DISCUSSI0N

DISCUSSION

Familial amyloidotic polyneuropathy is related to single transthyretin (TTR) amino acid substitutions, in which the mutated protein, synthesized mainly in the liver, leads to extracellular amyloid fibril deposition. Liver transplant is the only efficient and currently available therapy for FAP.

In this work we describe oral manifestations in Portuguese FAP patients, focusing on:

1. the study of labial salivary glands histology and their relations with the amyloid deposition;
2. the study of saliva composition;
3. the study of salivary flow rate that is diminished in the advanced patients;
4. the search for salivary biomarkers and their relations to the disease.

So far, the descriptions found in literature did not address these aspects of the disease, except for the study of Johansson et al in 1992 that investigated FAP patients in Sweden, along with saliva secretion rate and composition, and other factors related to the risk of dental caries.

Clinical findings

In previous reports (Johansson I et al 1992) thirty patients (16 males and 14 females) with a mean age ±SD of 60.2±10.3 years were studied. Nevertheless, only 23 patients completed the study. Fourteen patients could walk without a walking stick or crutches and nine used a wheelchair. 57% of the Sweden FAP patients were dentate, 43% were edentulous and wore full dentures. 77% of the patients had one or more moderate-to-large cavity caused by dental caries. There were no references to xerostomia, macroglossia or periodontal disease.

Another published case shows that patients with other forms of secondary amyloidosis have macroglossia, enlarged salivary glands, and xerostomia related to deposition of amyloid substance (Falk RH 1997).

Our study in Portuguese FAP patients involved 76 subjects and there were no complaints of xerostomia or difficulties in eating, swallowing, tasting or speaking. We have not seen any patient with candidiasis or atypical dental caries

(in cervical, incisal surfaces or cusp tip locations). Some patients had tooth loss and marginal gingivitis caused by dental plaque. There was no clinical evidence of amyloid deposition in the oral cavity, namely the presence of macroglossia.

The mean age of the patients ±SD (41 males and 35 females) was 37.1±10.4 years (range: 24–73 years). Male patients had a mean age 37.6±10.2 years (range: 27–68) and female patients had a lower mean age 36.6±10.7 years (range: 24–73).

There are some aspects that could explain the differences between our data and Johansson series:

1. mean age of FAP patients in our samples is lower;
2. in our sample FAP patients had a better neurological status;
3. the absence of xerostomia may be explained by the absence of severely affected patients in our sample;
4. we have not seen any patient with atypical dental caries (in cervical, incisal surfaces or cusp location) that are seen in oral cancer patients receiving radiotherapy and related to xerostomia (Meurman JH 2010). These rampant caries are seen in more FAP advanced disease stages;
5. in our sample of FAP patients the periodontal disease is infrequent and clinical irrelevant (marginal gingivitis). In Johansson study there are no references to the periodontal disease. In our sample the disease stage is not so disabling comparing to the FAP patients studied by Johansson, and patients can use their hands properly, which facilitates oral hygiene and tooth brushing, thus leading to a better oral condition.

Labial salivary gland biopsy

To identify amyloid deposition, the sampling of subcutaneous abdominal fat has been advocated as the simplest form of tissue biopsy (Libbey CA et al 1983). A fat aspirate stained with Congo red will be positive in 85% of patients with AL amyloidosis (Falk RH et al 1997).

Rectal biopsy has also been advocated for obtaining tissue samples for amyloid detection (Reed SB and Morris GT 1992), but this represents a more invasive procedure associated with a greater morbidity.

Amyloid deposition in the oral cavity has been the subject of controversy for many years. In 1947, Selikoff and Robitzek were the first to present evidence that gingival biopsy was of considerable value in the diagnosis of generalized secondary amyloidosis and found the gingival biopsy results to be positive in 14 of 18 cases studied.

The biopsy must be performed in gingival tissue approximately 4-mm wide, extending from the mucobuccal fold and including the interdental papilla, down to the periosteum in the maxillary canine/premolar region (Gorlin R and Gottsegen R 1949).

Lighterman I 1951 suggested that the gingival biopsy would be between 3-5 mm in length and taken from the posterior part of the maxilla.

Calkins and Cohen in 1960 reported that gingival biopsies were positive for amyloid in 5 of 8 cases of primary amyloidosis and 4 of 9 cases of secondary amyloidosis.

Trieger N et al in 1960 also expressed their appreciation in the use of gingival biopsy. Gingival tissues are not common and are of no diagnostic significance in the presence of amyloid (Lovett DW et al 1965). These findings supported that the blind biopsy of clinically normal gingival tissue is often unrewarding, being a high percentage of these biopsies unsuccessful in terms of demonstrating the presence of amyloid.

Other authors think that gingival biopsy may be preferable from a technical point of view and in terms of patient comfort (van der Waal et al 1973).

The tongue is the most frequently reported intraoral localization of amyloid deposition (van der Waal et al 1984). If the deposition is extensive, macroglossia may develop, and difficulty with speaking and chewing may ensue. Surgical management may be required if airway obstruction is anticipated (Mardinger O et al 1999).

If amyloidosis is the suspected diagnosis, a biopsy of the tongue must be performed (Keith DA 1972). However, other researchers have noted that the tongue biopsy is diagnostic in only 60% of the cases (Nandapalan V et al 1998).

In 1989, Delgado and Mosqueda have reported that the labial minor salivary glands are another intraoral site of amyloid deposition and thereby this represents another possible site for biopsy.

They compared gingival biopsy specimens with minor labial salivary gland biopsy specimens in 19 patients with evidence of secondary amyloidosis. They found that all 19 salivary gland specimens had some form of amyloid deposition,

whereas only 3 gingival biopsy specimens showed evidence of amyloid deposition.

The buccal mucosa represents an area of rapid cell turnover that will result in faster healing, thus providing easy access for a biopsy specimen. In addition, amyloid deposition was verified in 88% of the tissue specimens in the subepithelial connective tissue, regardless of the intraoral site from which they were obtained. This represents a depth of 1 to 3 mm required for a biopsy specimen needed to obtain a diagnosis of amyloidosis. The implications of these findings may result in a change in which intraoral biopsies for the diagnosis of amyloid are one of the best options to be performed. (Stoopler ET et al 2003).

To establish a definitive diagnosis of generalized amyloidosis, several tissues samples were used along the years. In our Hospital, the nerve biopsy was initially used to demonstrate amyloid deposition and is considered the gold standard procedure to identify amyloid deposition in FAP. Later, skin biopsy was preferred, as it was an easier and less traumatic method, providing reliable results as long as sweat glands, erector pili muscles, vessels and nerve fascicles were included in the sample (Guimarães A et al 1998; Gabriel CM et al 2000). In our Hospital the initial studies were performed in nerve biopsy, in skin biopsies and later in labial salivary glands.

In our study, labial salivary gland biopsy was carried out in 76 FAP patients. All subjects had the TTRVal30Met mutation determined by DNA analysis and a neurological observation to establish the medical history and define the clinical stage, before liver transplant or in patients under experimental therapeutic studies.

LSG biopsy was performed in all patients with only minimal and transitory morbidity and proved to be a high sensitivity method to detect amyloid deposition (91%). The finding that, among 11 asymptomatic carriers, one had light amyloid deposits and another had moderate amyloid deposits indicate a very good sensitivity of these surgical procedure, that results in a quicker healing time and increased patient comfort. Only one patient developed a small retention mucous cyst that disappeared spontaneously after 40 days. No patient had long-term secondary effects.

Histopathological changes in labial salivary glands

One of the questions that this thesis proposed to answer is the description of the histological changes in labial salivary glands and its relationship with TTR amyloid deposits.

Amyloid deposition is identified by Congo red staining and was always observed in polarized light to confirm the characteristic apple green birefringence.

Biopsy of labial salivary glands (LSG), proposed by Delgado and Mosqueda in 1989, revealed a great sensitivity and specificity that was considered as the reference diagnostic method for diagnosis of systemic amyloidosis. In 1993, Hachulla et al suggested that LSG biopsy can be used to assess systemic amyloid and light chain amyloid amyloidosis.

In 1989, Delgado and Mosqueda studied 19 patients with clinical findings suggestive of secondary amyloidosis and compared the results with LSG and gingival biopsies. Amyloid deposits were present in all salivary gland biopsies with the following distribution: periductal involvement was found along the basement membrane in all salivary glands samples; 16 cases had periacinar infiltration, 13 cases had perivascular infiltration and 7 cases showed interstitial fibrosis.

Labial salivary gland biopsy was studied for diagnosis of FAP amyloid polyneuropathy (Lechapt E et al 1999). Amyloid deposits were detected in 7 of the 32 patients with axonal polyneuropathy, were abundant in 4 patients, moderate in 2, and mild in 1. They were detected predominantly around acini (n=1), around excretory ducts (n=4), in interstitium (n=1) in vessel walls (n=1) and consistent with transthyretin (TTR) amyloidosis in 5 of 7 patients, 3 Portuguese and 2 French, none of whom had a family history of neuropathy.

Our study involved 76 patients (41 men, 35 women) and 11 asymptomatic carriers (6 men, 5 women). All subjects had the TTRVal30Met mutation determined by DNA analysis and a neurological observation to establish the medical history in order to define the clinical stage.

In 16 subjects, the gland morphology was preserved without amyloid deposition. In 12 subjects, the global gland morphology was normal; however, we could observe very sparse amyloid deposits in the connective tissue, in the adventitia of the arterioles or in the proximity of the basement membrane embracing some glandular acini. In 10 individuals, the global glandular aspect

was preserved; we could see mild interstitial septal fibrosis and more pronounced amyloid deposition surrounding several glandular acini and excretory ducts.

In 49 subjects, the global glandular aspect was severely changed with evident loss of glandular acini, marked interstitial fibrosis and hyalinization of interstitial tissue and occasional lipidic substitution with profuse amounts of amyloid. The immunohistochemistry study using the antibody anti-TTR confirmed that the main component of amyloid is transthyretin in all samples with amyloid deposition. With this method, the distribution of amyloid deposits was similar to the findings obtained with Congo Red. Our data are similar to those described in the literature.

In 13 cases, the amyloid deposits were also studied by transmission electron microscopy (EM), in order to establish their ultrastructural characteristics. Amyloid fibrils were identified in all cases studied with EM. We have not seen significant ultrastructural changes in the glandular acini or excretory ducts cells. In the glands with more advanced lesions, increased amounts of collagen bundles surrounding the glandular acini were seen. The amyloid substance appeared as bundles of fine straight fibrillar material, with 8 nm of diameter, whose size, orientation and organization was variable. This fibrillar material was among the collagen bundles but, here and there, it was also in direct contact with the basal lamina surrounding the glandular acini and the excretory ducts. These results are similar to those described by Delgado et al in 1997.

Histopathological changes and disease stage

Distal motor latency, motor conduction velocity (segment between fibula and ankle) and M-wave amplitude (peak-to-peak) of the right peroneal nerve (recorded from the extensor digitorum brevis) and the sensory nerve action potential of the right sural nerve (that record its amplitude and conduction velocity), have been postulated as a standardized neurophysiological study to examine progression of the neuropathy and to help decision for better option treatment (Conceição I et al 2007).

To answer this problem, one of the hypotheses suggested in this thesis is that histological changes of labial salivary glands could be used as a prognostic factor of disease progression. We compared the histological changes of LSG with the different stages of the disease. The comparison between LSG histology and

the clinical condition shows that 63% of the patients (48/76) and one asymptomatic carrier had abundant amyloid deposits and gland destruction. These severe changes were found in 51% of stage 1 patients, in 64% of stage 2 patients and in all stage 3 patients. Moderate deposition of amyloid was seen in four of stage 1 patients and six of stage 2 patients. Slight amyloid deposition with normal gland morphology was restricted to stage 1 patients (n=11) and one asymptomatic carrier. Seven patients in stage 1, between the second and the fourth year of symptomatic disease, had normal biopsies, without amyloid deposition.

These data should be interpreted with caution, given the wide variability of amyloid deposits in different stages of the disease and the small size of the sample. Nevertheless, it must be underlined that 100% of patients with moderate disease have a positive biopsy. The finding that, among 11 asymptomatic carriers, one had light amyloid deposits and another had moderate amyloid deposits and some degree of glandular destruction, indicates that the pathogenic process starts (and may even progress significantly) before clinical symptoms appear.

In 2011, a study performed at Unidade Clínica de Paramiloidose in Hospital Santo António in Porto, comparing the sensitivity of the labial salivary gland biopsy with electromyography performed in patients with FAP and showing conducting abnormalities of small sensory fibers, showed that the labial salivary gland biopsy is more sensitive in detecting early stages of the disease (unpublished data).

In our hospital, immunocytochemistry of TTR is only carried out for diagnostic study when there was not a molecular genetic diagnosis of FAP. The role of neuropathy – which causes denervation of glandular structures - and the role of amyloid infiltration, which causes tissue replacement with fibrosis, in the loss of saliva secretion are yet to be established. In these biopsies, we confirmed that amyloid deposition leads to a significant loss of glandular tissue even in the first years of the disease, but the remaining acini and ducts have a normal ultrastructural appearance. This finding may help to explain the maintenance of a minimal saliva secretion until the late stages of the disease. Our data support previous histopathological and ultrastructural findings (Delgado W et al 1997).

The possibility that oligomeric intermediate may be present in labial salivary glands biopsies and could contribute to the salivary gland dysfunction, could only be demonstrated using more specific laboratory techniques such as

mass spectrophotometry, which is not available at our hospital. Laser microdissection with mass spectrometry (LMD/MS) with customized bioinformatic assessment of the constituents of the Congophilic deposits is now the gold standard for typing amyloid enabling precise identification of type in over 98% of cases (Cohen AD and Comenzo RL 2010).

Salivary hypofunction

Clinical experience of more than 20 years with FAP patients has alerted us to the fact that patients in the earliest stages of the disease had the same propensity for caries than others individuals, being xerostomia an uncommon complaint (unless directly asked for that symptom). Hyposalivation was more evident in later stages of the disease with devastating consequences for dental health and development of rampant caries. We then tried to conduct a qualitative and quantitative study of saliva to compare it with a group of healthy volunteers.

To understand the influence of the disease in the development of oral pathology, we systematically studied a group of 39 patients in the different stages of the disease, the majority of which were evaluated in the context of their preparation for liver transplantation.

Johansson et al in 1992 studied a group of thirty patients in the Skellefteå – Sweden, concerning saliva secretion rate and composition and other factors related to the risk of dental caries in patients with various degrees of FAP. They conclude that salivary hypofunction frequently develops in patients with FAP and that the level of mouth dryness is correlated to the degree of the disease.

Our data confirms the results of Johansson et al in 1992. The mean flow rate was 6.45±2.83 cm in FAP patients and 7.22±3.0 cm in the control group. When we analyzed the degree of the disease as a covariate factor, significant differences between the FAP0 and FAP3 were found with lower mean salivary flow in more advanced disease. In our study we compare other salivary parameters that could influence salivary flow such as, age, sex, temperature, humidity, caries index and BMI. We only find BMI as a statistically significant difference for male sex FAP patients (p=0.00).

These data support that xerostomia in Portuguese FAP patients must be included in the number of genetic or other chronic diseases, that may be associated with salivary dysfunction (von Bültzingslöwen I et al 2007). Nevertheless, our data must be interpreted with caution as the mean age of FAP

patients in our sample is lower than those in Johansson study and the methodology used was different.

Risk for developing oral pathology

A variable number of factors are known to be a potential risk for developing dental caries as a consequence of salivary secretion rate: composition and buffer capacity, number of cariogenic microorganisms, intake of fermentable carbohydrates, dental hygiene, fluoride exposition and oral motor function. Studies show that more than two negative factors cause a significant increase in caries development (Sanchéz-Pérez L et al 2009).

Several salivary parameters are studied by Johansson in Swedish patients: unstimulated and stimulated saliva, salivary flow rate, concentration of total protein, thiocyanate, amylase, lysozyme, secretory IgA, salivary peroxidase pH, buffer capacity, hexosamines, fucose, sialic acid, sodium, potassium, calcium and phosphate.

In Johansson study, the FAP group had higher salivary concentration of total protein, amylase, lysozyme, secretory IgA, potassium and phosphate than the control group.

Our data shows that for sodium, lysozyme, IgG and IgM, the values were inferior to the lower limit of the method, so we divide all those salivary parameters in two categories. The sodium values equal or superior to 21 mmol/L were found in the FAP group. Lysozyme was divided in three categories. The values between 6 mg/L and 12 mg/l and inferior to 6.0 mg/L are more frequent in FAP patients. The bicarbonates values are higher in the control group ($p=0.00$). We found a statistically significant difference for the pH in two groups ($p=0.00$). A statistically significant positive correlation ($p=0.00$) was found between the bicarbonates and the pH. As the bicarbonates increases the salivary pH returns more alkaline. As for the Johansson's study, the highest values of proteins were found in FAP patients ($p=0.03$). Albumin particularly presented higher values and was statistically significant ($p=0.03$) in FAP patients.

In the FAP patients group we found a positive and statistically significant correlation ($p=0.00$) between proteins and the osmolarity. Together with the bicarbonates, they contribute to a regulatory effect on the pH providing saliva alkalinity, which may explain the low CPO index of the FAP patients when

compared with the control group. The mean salivary value of ammonia is similar in both groups.

A statistically significant positive correlation (p=0.01) was found between the ammonia and the CPO index in the group of FAP patients.

For the IgA there is also a statistically significant difference (p=0.05) between two groups with highest values in group of FAP patients, which suggests a protective effect for the development of caries and other pathologies, namely oral candidiasis.

Johansson's study could not evaluate whether patients with FAP had more dental caries than other individuals, because there were few dentate patients distributed into each sex and age stratum. The data could not be merged because age and sex are important covariates for the incidence of dental caries. That study makes no reference to the presence of oral candidiasis in patients with FAP.

It is noteworthy that in our sample both the mean age of FAP patients and the methodology employed was different. Therefore our results must be interpreted with caution.

Salivary biomarkers

The product obtained in labial salivary gland biopsies has been used for the studies that are in course in the Amyloid Unity of IBMC, supervised by Professor Maria João Saraiva, about the pathogenesis of FAP. It was proposed to me, as part of this work, to study the biological expression of two biomarkers (MMP-9 and ZAG) in healthy volunteers and FAP patients to determine their relationship with the disease staging.

MMP-9 and salivary glands

In salivary glands the basal lamina in acini is connected to the cytoskeleton of acinar cells via integrins in the basal plasma membrane (Yeaman C et al 1999). Since specific matrix metalloproteinases (MMPs), in particular MMP-2, MMP-3, MMP-9 degrade basal lamina components, and patients with primary SS show elevated levels of MMP-9 in saliva, it has been hypothesized that MMPs might be involved in the loss of cell anchorage to the basal lamina (Konttinen YT et al 1998).

In a previous study we showed that MMP-9 occurs as a complex with neutrophil gelatinase-associated lipocalin (NGAL); both molecules were found increased in FAP and in vitro degraded TTR aggregates and fibrils. Biglycan, NGAL and MMP-9 are transcriptionally up-regulated by NF-kB - a transcription factor that is activated in FAP nerves and salivary glands (Sousa MM et al 2004).

This study confirms that MMP-9 is expressed in acinar and ductal cells of labial salivary glands and shows that MMP-9 levels are present in different stages of the disease. However, we only found statistically significant differences in more advanced disease staging.

Similar studies should be performed with a larger sample of patients to confirm the data presented here.

ZAG and salivary glands

Zinc-alpha-2-glycoprotein (ZAG) is a member of the immunoglobulin superfamily with high degrees of sequence similarity to class-1 major histocompatibility complex antigens. It has been detected in several human body fluids, including whole saliva (Vitorino R et al 2005) and parotid saliva (Hardt M et al 2005). Immunohistochemical analysis localized this glycoprotein to the serous cells of the parotid and submandibular glands (Tada T et al 1991).

In FAP patients, the presence of salivary ZAG is shown by Macedo B et al in 2007.

This work demonstrates for the first time the presence of ZAG in FAP labial salivary glands and their immunochemical localization, which is similar to those seen in normal salivary glands (Tada T et al 1991).

In this present study, glandular ZAG was expressed in different stages of the disease, and their levels are higher in more advanced stages of disease.

Also higher levels of salivary ZAG were found in more advanced stages of disease.

Once ZAG has been described as associated with cachexia in cancer patients, further investigations in FAP patients may be useful to understand the cachexia that FAP patients present in more advanced stages of the disease.

VII: CONCLUSIONS

CONCLUSIONS

We initiated this work at the Hospital of Santo Antonio in Porto about 20 years ago. We started to perform a biopsy of labial salivary glands instead of nerve biopsy, since it entails some risk of morbidity for patients.

The review of the literature carried out showed the high sensitivity of the biopsy of labial salivary glands for the detection of amyloid deposits, also demonstrating the association of amyloid deposits with the TTR. The results obtained and expressed in this thesis enabled us to achieve the first goal of this work.

We studied the frequency of amyloid deposits and characterized their distribution in the labial salivary glands. Then we related the amyloid deposits with the stages of the disease, therefore accomplishing two of our goals.

The observation of patients with FAP has shown that young patients had dental caries. However, they are not as important as we have seen in patients in more advanced stages of the disease with an increased number of cavities, as it presented with appearance of large coronary destruction and objective observation of dry oral mucosa which was not referred by the patients. Then, we decided to study quantitative and qualitative aspects of saliva, as well as the existence of hyposalivation, having thus fulfilled another objective of this work.

FAP is a genetic disorder in which individuals sooner or later will have knowledge of. We asked about the importance of an early sign of changes in saliva, in order to prevent the onset of the consequences associated with it, in particular those concerning the emergence of infectious diseases and prevention of caries. We set out to study some biomarkers including MMP-9 and ZAG to get information, in order to achieve some correlation with early changes of the salivary glands.

In a previous study in FAP labial salivary glands, it was demonstrated that MMP-9 is over expressed. In this work we demonstrate that MMP-9 is expressed in all disease stages and higher values are present in more advanced disease. Hence, MMP-9 may contribute to explain de xerostomia presented by these patients.

As for ZAG, this is the first work done in these patients. Hence, other studies should be carried out, in order to understand the true meaning of its expression in the salivary glands and its role in these histopathological changes.

VIII: FUTURE PROSPECTS

Future Prospects

This study was conducted in a clinical setting, for a better understanding of oral manifestations in a group of FAP patients whose average age is lower than in the Norwegian study. We suggest some research hypotheses:

- to study the mechanisms involving xerostomia mainly the changes in aquaporins;
- to study the salivary alpha-amylase as a biomarker for the sympathetic nervous system;
- to perform labial salivary glands biopsy as a method of choice to evaluate recovery of post-transplanted patients and evaluation of therapeutic studies.

IX. SUMMARY

SUMMARY

Familial amyloid polyneuropathy is an inherited form of amyloidosis first described by Corino de Andrade in families from northern Portugal. The main clinical feature is a highly disabling ascending autonomic, sensory and motor neuropathy that is fatal, on average, 11 years after onset.

Autonomic neuropathy is responsible for severe gastrointestinal, cardiocirculatory and genitourinary problems. Renal, ocular and myocardial involvement may also occur in some patients.

The disease is due to deposition of a variant protein transthyretin as amyloid substance. TTRVal30Met is by far the most common and the one responsible for the large foci identified in Portugal, Sweden, Majorca and Japan.

Nowadays, the diagnosis of FAP TTR Val30Met is based on molecular analysis of the TTR mutation.

Demonstration of amyloid deposition remains the gold standard for the diagnosis of the onset of the disease, particularly when invasive procedures such as liver transplantation or the inclusion of patients in clinical trials are proposed as a treatment.

In our Hospital, the nerve biopsy was initially used to demonstrate amyloid deposition and was considered the best procedure to identify amyloid deposition in FAP patients. Later skin biopsies, rectal biopsies were also used.

The historical review of the disease and its clinical manifestations are described in Chapter II.

In present study, labial salivary glands biopsy was used as a less invasive method, in all patients with only minimal and transitory morbidity and amyloid deposition was detected in 69 of 76 patients (91%).

One of the hypotheses suggested in this thesis is that histological changes of labial salivary glands could be used as a prognostic factor of disease progression. The results, despite the sensitivity of the method do not allow for this correlation. Nevertheless it must be underlined that 100% of patients with moderate disease have a positive biopsy. The finding that, among 11 asymptomatic carriers, one had light amyloid deposits and another had moderate amyloid deposits and some degree of glandular destruction,

indicates that the pathogenic process starts (and may even progress significantly) before clinical symptoms appear.

Pathological data from labial salivary glands biopsy were correlated with some clinical aspects, emphasizing the relations between that data the oral problems, amyloid deposition and clinical stage of the disease. The results are presented in Chapter IIi as a scientific paper: “Usefulness of labial salivary biopsy in familial amyloid polyneuropathy Portuguese type”.

Clinical experience of more than 20 years with FAP patients, has alerted us to the fact that patients in the earliest stages of the disease had the same propensity for caries than others individuals, being xerostomia an uncommon complaint (unless directly asked for that symptom).Then we conduct a qualitative and quantitative study of saliva to compare it with a group of healthy subjects. This is the first study of clinical findings, oral complaints, saliva secretion rate and composition in FAP Portuguese patients. Our study in Portuguese FAP patients involved 37 subjects (Chapter IV).

In an attempt to find a reliable biological marker of the disease onset, in Unidade Amilóide do Instituto de Biologia Molecular e Celular do Porto, a previous study showed that MMP-9 was increased in FAP salivary glands. A new study was done to find if MMP-9 is expressed in saliva and salivary gland in different stages of the disease.

Another marker that we studied before in saliva was ZAG. In present study, we study also glandular ZAG in different disease staging.

The previous results of these salivary and glandular markers are described in Chapter V.

X. RESUMO

RESUMO

A Polineuropatia amiloidótica familiar é uma forma hereditária de amiloidose primeiramente descrita por Corino de Andrade em famílias do norte de Portugal. Caracteriza-se principalmente por uma neuropatia autonómica sensorial e motora ascendente, altamente incapacitante que é fatal, em média, 11 anos após o início.

A neuropatia autonómica é responsável por alterações graves dos aparelhos cardiocirculatório, gastrointestinal e genito-urinário. O envolvimento ocular, renal e do miocárdio também pode ocorrer em alguns doentes.

A doença é devida à deposição como substância amilóide de uma variante da transtirretina. A TTRVal30Met é de longe a mais comum e uma das responsáveis pelos grandes focos identificados em Portugal, Suécia, Japão e Maiorca.

Actualmente o diagnóstico da PAF Val30Met é realizado por análise molecular da TTR mutante.

A demonstração da substância amilóide continua sendo o "gold standard" para o diagnóstico do início da doença, especialmente quando são propostos como tratamento, procedimentos invasivos como transplante de fígado ou a inclusão de doentes em ensaios clínicos.

No nosso hospital a biopsia do nervo foi utilizada inicialmente para demonstrar a presença de substância amilóide e era considerado o melhor procedimento para a sua identificação nos doentes com PAF. Mais tarde realizaram-se também biopsias da pele e do recto. Uma revisão histórica da doença e das suas manifestações clínicas é descrita no Capítulo II.

No presente estudo foi usada a biopsia das glândulas salivares labiais como um método menos invasivo e com menor morbilidade do que a biopsia do nervo, e a substância amilóide foi encontrada em 69 dos 76 doentes estudados (91%).

Uma das hipóteses sugeridas nesta tese é a que as alterações histológicas das glândulas salivares labiais pode ser usada como um factor de prognóstico de progressão da doença, Os resultados obtidos, apesar da sensibilidade do método não permitem tirar esta correlação. No entanto devemos sublinhar que 100% dos doentes com doença moderada têm uma biopsia positiva. A constatação de que entre os 11 portadores assintomáticos,

um tinha depósitos ligeiros de amilóide e outro tinha depósitos moderados de amilóide com algum grau de destruição glandular, revela que o processo patogénico começa (e pode progredir muito) antes do aparecimento da sintomatologia clínica.

Os resultados histológicos obtidos nas glândulas salivares labiais foram correlacionados com alguns aspectos clínicos, particularmente com especial relevo entre a deposição de substância amilóide, os problemas orais e o estádio clínico da doença. Os resultados são apresentados no Capítulo III sob a forma de artigo científico: "Usefulness of labial salivary gland biopsy in familial amyloidotic polyneuropathy Portuguese type".

A experiência clínica de mais de 20 anos com doentes com PAF, alertou-me para o facto daqueles nos estádios iniciais da doença, apresentarem a mesma propensão para a cárie que os indivíduos saudáveis. A xerostomia apenas era referida quando perguntávamos directamente ao doente se tinha a boca seca. Procurei então realizar um estudo qualitativo e quantitativo da saliva destes doentes e comparar os resultados com um grupo de indivíduos saudáveis. Este é o primeiro trabalho realizado em doentes com PAF. Envolveu 37 doentes e os seus resultados são descritos no Capítulo IV.

Na tentativa de encontrar um marcador biológico confiável capaz de identificar os estádios precoces da doença, um estudo anterior realizado no Instituto de Biologia Molecular e Celular do Porto, mostrou que a MMP-9 é expressa na saliva e nas glândulas salivares labiais dos doentes com PAF. Neste trabalho estudámos a expressão da MMP-9 na saliva e nas glândulas salivares labiais em diferentes estádios da doença. Outro marcador previamente estudado na saliva, na mesma Instituição foi a ZAG. Neste trabalho estudámos estes marcadores na saliva e nas glândulas salivares labiais e os seus resultados são descritos no Capítulo V.

XI. REFERENCES

REFERENCES

Adams, W. 1872. Mollities ossium. Transactions of the Pathological Society of London, 23, 186-188.

Agha-Hosseini, F., Mirzaii-Dizgah I., Rahimi A. 2009. Correlation of serum and salivary CA15-3 levels in patients with breast cancer Med Oral Patol Oral Cir Bucal. Oct 1; 14 (10):e521-4.

Agostini, M., Tucci P., Melino G. 2011. Cell death pathology: Perspective for human diseases. Biochem Biophys Res Commun. Oct 28; 414(3):451-5..

Ahn, S.J, Ahn S.J, Wen Z.T, Brady L.J, Burne R., A. 2008. Characteristics of biofilm formation by Streptococcus mutans in the presence of saliva. Infect Immun. Sep; 76(9):4259-68.

Alevizos, I., Alexander S., Turner R., J., Illei G., G. 2011.MicroRNA expression profiles as biomarkers of minor salivary gland inflammation and dysfunction in Sjögren`s Syndrome. Arthritis Rheum. February; 63(2), 535–544.

Al-Hashimi, I., Levine M., J. 1989. Characterization of *in vivo* salivary-derived enamel pellicle. Arch Oral Biol. 34(4):289-95.

Almståhl, A., M., Wikström, J., Groenink J. 2001.Lactoferrin, amylase and mucin MUC5B and their relation to the oral microflora in hypossalivation of different origins. Oral Microbiol Immunol.16: 345–352.

Almståhl, A., Wikström M. 2003.Electrolytes in stimulated whole saliva in individuals with hyposalivation of different origins. Arch Oral Biol.48: 337-344.

Alves, M., Conceição I., Luis M., L. 1997. Neurophysiological evaluation of sexual dysfunction in familial amyloidotic polyneuropathy--Portuguese type. Acta Neurol Scand. Sep; 96(3):163-6.

Amano, A., Sojar H., T., Lee J., Y., Sharma A., Levine M., J., Genco R., J.1994. Salivary receptors for recombinant fimbrillin of Porphyromonas gingivalis. Infect Immun. Aug; 3372-3380.

Ambudkar I., S. 2011. Dissection of calcium signaling events in exocrine secretion. Neurochem Res. Jul; 36(7):1212-21.

Ancsin, J., B. 2003. Amyloidogenesis: historical and modern observations point to heparan sulfate proteoglycans as a major culprit. Amyloid. Jun; 10(2):67-79.

Andersson, R. 1970. Hereditary amyloidosis with polyneuropathy. Acta Med Scand. Jul- Aug; 1-2 (1):85-94.

Andersson, R. 1976. Familial amyloidosis with polyneuropathy. A clinical study based on patients living in northern Sweden. Acta Med Scand Suppl. 590:1-64.

Ando, Y., Yi S., Nakagawa T., Ikegawa S., Hirota M., Miyazaki A., Araki S., I. 1991. Disturbed metabolism of glucose and related hormones in familial amyloidotic polyneuropathy: hypersensitivities of the autonomic nervous system and therapeutic prevention. Auton Nerv Syst. Jul; 35(1):63-70.

Ando, Y., Nyhlin N., Suhr O., Holmgren G., Uchida K., Sahly M., E.,Yamashita T., Terasaki H., Nakamura M., Uchino M., Ando M. 1997.Oxidative Stress Is Found in Amyloid Deposits in Systemic Amyloidosis. Biochem Biophy Res Commun. 232**;** 497-02.

Ando, Y., Nakamura M., Araki S. 2005.Transthyretin-Related Familial Amyloidotic Polyneuropathy. Arch Neurol; 62:1057-106.Review.

Andrade C.1952. A peculiar form of peripheral neuropathy; familiar atypical generalized amyloidosis with special involvement of the peripheral nerves. Brain. 75; 408-427.

Andrade, M., J. 2009. Lower urinary tract dysfunction in familial amyloidotic polyneuropathy, Portuguese type. Neurourol Urodyn. 28(1): 26-32.

Angiero, F., Seramondi R., Magistro S., Crippa R., Benedicenti S., Rizzardi C., Cattoretti G. 2010. Amyloid deposition in the tongue: clinical and histopathological profile. Anticancer Res. Jul; 30(7):3009-14.

Anil, S., Ellepola A., N., Samaranayake L., P. 2001. Post-antifungal effect of polyene, azole and DNA-analogue agents against oral Candida albicans and Candida tropicalis isolates in HIV disease. J Oral Pathol Med. Sep; 30(8):481-8.

Apitz, K. 1940. Die Paraproteinosen (Über die Störung des Eiweisstoffwechsels bei Plasmocytom). Virchows Archiv für Pathologische Anatomie und Physiologie (B); 306; 631-639.

Araky, S., Shigehiro Y. 2000. Pathology of familial amyloidotic polyneuropathy with TTR Met 30 in Kumamoto, Japan. Neuropathology. 20, (Suppl) S47-S51.

Aubert, B., Cochet C., Souvignet C., Chambaz E.., M. 1990. Saliva from cystic fibrosis patients contains an unusual form of epidermal growth factor. Biochem Biophys Res Commun. Aug 16; 170(3):1144-50.

Bao, Y., Bing C., Hunter L., Jenkins J., R., Wabitsch M., Trayhurn P. 2005. Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed and secreted by human (SGBS) adypocites. FEBS Lett. Jan 3; 579(1):41-7.

Bardow A., Hofer E., Nyvad B., ten Cate J.,M., Kirkeby S., Moe D., Nauntofte B.2005. Effect of saliva composition on experimental root caries. Caries Res. 39(1); 71-7.

Beirão, I., Lobato L., Costa P.,M., Fonseca I., Mendes P., Silva M., Bravo F., Cabrita A., Porto G. 2004. Kidney and anemia in familial amyloidosis type I. Kidney Int. Nov; 66(5):2004-9.

Ben-Aryeh, H., Serouya R., Kanter Y., Szargel R., Laufer D. 1993.Oral health and salivary composition in diabetic patients. J Diabetes Complications. Jan-Mar; 7(1); 57-62.

Bennhold, H. 1922. Specific staining of amyloid by Congo red. München Med Wochnschr. 69:1537-1538.

Benson, M., D., Dwulet F., E. 1985. Identification of carriers of a variant plasma prealbumin (transthyretin) associated with familial amyloidotic polyneuropathy type I. J Clin Invest. Jan; 75(1):71-5.

Benson, M., D., Wallace M., R., Tejada E.., Baumann H. Page B. 1987. Hereditary amyloidosis: description of new American kindred with late onset cardiomyopathy. Appalachian amyloid. Arthritis Rheum. Feb; 30(2):195-200.

Benson, M., D., Liepnieks J., Uemichi T., Wheeler, G. Correa R. 1993. Hereditary renal amyloidosis associated with a mutant fibrinogen alpha-chain. Nat Genet. Mar; 3(3): 252-255.

Benson, M., D., Liepnieks J., J., Yazaki M., Yamashita T., Hamidi Asi K., Guenther B., Kluve-Beckerman B. 2001. A new human hereditary amyloidosis: the result of a stop-codon mutation in the apolipoprotein AII gene. Genomics. Mar; 72(3):272-277.

Biewend, M., L., Menke D., M., Calamia K., T. 2006. The spectrum of localized amyloidosis: a case series of 20 patients and review of the literature. Amyloid. Sep; 13(3):135-42.

Bing, C., Bao Y., Jenkins J., Sanders P., Manieri M., Cinti S., Tisdale M.,J., Trayhurn P. 2004. Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed in adypocites and is up-regulated in mice with cancer cachexia. Proc Natl Acad Sci U S A. Feb 24; 101(8):2500-5.

Blake, C., C., Geisow M., J., Swan I., D., Rerat C., Rerat B.1974. Structure of human plasma prealbumin at 2-5 A resolution. A preliminary report on the polypeptide chain conformation, quaternary structure and thyroxine binding. J Mol Biol. Sep 5; 88(1):1–12.

Bonaïti, B., Olsson M., Hellman U., Suhr O., Bonaïti-Pellié C., Planté-Bordeneuve V. 2010.TTR familial amyloid polyneuropathy: does a mitochondrial polymorphism entirely explain the parent-of-origin difference in penetrance? Eur J Hum Genet. Aug; 18(8): 948-952.

Bots, C.,P., Brand H.,S., Veerman E.,C,, Korevaar J.,C., Valentijn-Benz M., Bezemer P.,D., Valentijn R.,M., Vos P.,F., Bijlsma J.,A., ter Wee P.,M,, Van Amerongen B.,M., Nieuw Amerongen A.,V. 2005. Chewing gum and a saliva substitute alleviate thirst and xerostomia in patients on haemodialysis. Nephrol Dial Transplant. Mar; 20(3):578-84.

Boutaga, K., Savelkoul P., H., Winkel E., G., van Winkelhoff A., J. 2007.Comparison of subgingival bacterial sampling with oral lavage for detection and quantification of periodontal pathogens by real-time polymerase chain reaction. J Periodontol. Jan; 78(1):79-86.

Boyce, H., W., Bakheet M., R. 2005. Sialorrhea: a review of a vexing, often unrecognized sign of oropharyngeal and esophageal disease. J Clin Gastroenterol. Feb; 39(2):89-97.

Bratthall, D., Widerström L. 1985. Ups and downs for salivary IgA. Scand J Dent Res. Apr; 93(2):128-34.

Brysk, M.,M., Lei G., Rajaraman S., Selvanayagam P., Rassekh C.,H., Brysk H., Tyring S.,K., Arany I. 1997. Gene expression of zinc-alpha 2-glycoprotein in normal human epidermal and buccal epithelia. In Vivo. May-Jun; 11(3):271-4.

Bucciantini, M., Giannoni E., Chiti F., Baroni F., Formigli L., Zurdo J., Taddei N., Ramponi G., Dobson C., M., Stefani M.2002. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature April 4; 416(6880):507-511.

Bucciarelli, L., G., Wendt T., Rong L.., Lalla E., Hofmann M., A., Goova M., T., Taguchi A., Yan S., F., Yan S., D., Stern D.,M., Schmidt A., M. 2002. RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. Cell Mol Life Sci. Jul; 59(7):1117-28.

Bundred, N., J., Miller W., R, Walker R., A. 1987. An immunohistochemical study of the tissue distribution of the breast cyst fluid protein, zinc alpha 2 glycoprotein. Histopathology. Jun; 11(6):603-10.

Bürger, R., Hahnel S., Reichert T., E., Rosentritt M., Behr M., Gerlach T., Handel G., Gosau M. 2010. Adhesion of Candida albicans to various dental implant surfaces and the influence of salivary pellicle proteins. Acta Biomater Jun; 6(6):2307-13

Bürgi, W., Schmid K. 1961. Preparation and properties of Zn-alpha 2-glycoprotein of normal human plasma. J Biol Chem. Apr; 236:1066-74.

Bürgi, W., Simonen S., Baudner S., Schmid K. 1989. Unusually high concentrations of Zn alpha 2-glycoprotein and the lack of alpha 2HS-glycoprotein in human ejaculates. Clin Chem. Aug; 35(8):1649-50.

Calkins, E., Cohen A., S. 1960. Bull Rheum Dis. Apr; 10:215-8. Diagnosis of amyloidosis.

Cardoso, I., C., Goldsbury C., S., Müller S., A., Olivieri V., Wirtz S., Damas A., M., Aebi U., Saraiva M., J. 2002. Transthyretin fibrillogenesis entails the assembly of monomers: a molecular model for in vitro assembled transthyretin amyloid-like fibrils. J Mol Biol. Apr 12; 317(5): 683-95.

Cauwe, B., Van den Steen P., E., Opdenakker G. 2007. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. Crit Rev Biochem Mol Biol. May-Jun; 42(3):113-85.

Cecchi, C., Baglioni S., Fiorillo C., Pensalfini A., Liguri G., Nosi D., Rigacci S., Bucciantini M., Stefani M. 2005.Insights into the molecular basis of the differing susceptibility of varying cell types to the toxicity of amyloid aggregates. J Cell Sci. Aug 1; 118(Pt 15):3459-70.

Chamberlain, A., K., MacPhee C., E., Zurdo J., Morozova-Roche L., A., Hill H., A, Dobson C., M. et al. 2000.Ultrastructural organization of amyloid fibrils by atomic force microscopy. Biophys J; 79:3282-93.

Chicharro, J., L., Serrano V., Ureña R., Gutierrez A., M., Carvajal A., P Fernández-Hernando P., Lucía A. 1999. Trace elements and electrolytes in human resting mixed saliva after exercise Br J Sports Med. Jun; 33(3):204-7.

Chimenos-Kustner, E., Marques Soares M., S. 2002. Burning mouth and saliva. Med Oral Jul-Oct 7(4):244-53: Review.

Christersson, C., E., Lindh L., Arnebrant T. 2000. Film-forming properties and viscosities of saliva substitutes and human whole saliva Eur J Oral Sci; Oct; 108(5):418-25.

Chung, C., M., Connors L., H., Benson M., D., Walsh M., T. 2001. Biophysical analysis of normal transthyretin: implications for fibril formation in senile systemic amyloidosis. Amyloid Jun; 8 (2):75-83.

Coelho, T., Sousa A., Lourenço E., Ramalheira J.1994.A study of 159 Portuguese patients with familial amyloidotic polyneuropathy (FAP) whose parents were both unaffected J Med Genet. Apr; 31(4):293-299.

Cohen, A., D., Comenzo R., L. 2010. Systemic light-chain amyloidosis: advances in diagnosis, prognosis, and therapy. Hematology Am Soc Hematol Educ Program. 2010:287-94.

Cohen, A., S., Calkins E.1959.Electron microscopic observations on a fibrous component in amyloid of diverse origins. Nature. Apr 25; 183(4669), 1202-3.

Coimbra, A., Andrade C.1971a. Familial amyloid polyneuropathy: an electron microscope study of the peripheral nerve in five cases. I. Interstitial changes. Brain; 94(2):199 –206.

Coimbra, A., Andrade, C., 1971b. Familial amyloid polyneuropathy: an electron microscope study of peripheral nerve in five cases. II. Nerve fibril changes. Brain; 94(2): 207–212.

Conceição, I., M., Miranda L., C, Simões E., Gouveia R.,G., Evangelista T., D., de Carvalho M., A. 2005. Bone mineral density in familial amyloid polyneuropathy and in other neuromuscular disorders. Eur J Neurol. Jun; 12(6):480-2.

Conceição, I., De Carvalho M.2007.Clinical Variability in Type I Familial Polyneuropathy (Val30Met): Comparison between late and early-onset cases in Portugal. Muscle-Nerve Jan 35(1):116-8.

Connors L.,H., Lim A., Prokaeva T., Roskens V., A., Costello C.,E. 2003. Tabulation of human transthyretin (TTR) variants. Amyloid. Sep 10(3):160-84.

Contestabile, A., Ciani E. 2004. Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation. Neurochem Int. Nov; 45(6):903-14.

Corthésy, B., Spertini F.1999.Secretory immunoglobulin A: from mucosal protection to vaccine development. Biol Chem. Nov; 380(11):1251-62.

Costa, P., P., Figueira A., S., Bravo F., R. 1978. Amyloid fibril protein related to prealbumin in familial amyloidotic polyneuropathy. Proc Natl Acad Sci U S A Sep; 75(9):4499-503.

Coutinho, P., Martins da Silva A., Lopes Lima J., Resende Barbosa.1980. Forthy years of experience with type I amyloid neuropathy (review of 483 cases). Glenner GC, Costa PP, Freitas AF (eds). Amyloid and Amyloidosis. Excerpta Medica, Amsterdam, 88-98.

Cuida, M., Legler D., W., Eidsheim M., Jonsson R. 1997. Complement regulatory proteins in the salivary glands and saliva of Sjögren`s syndrome patients and healthy subjects. Clin Exp Rheumatol. Nov-Dec; 15(6):615-23.

Davies, A., Douglas L., Hendrich J., Wratten J., Tran Van Minh A., Foucault I. Koch D., Pratt W., S., Saibil H.,R. Dolphin A., C. 2006. The Calcium channel alpha2delta-2 subunit partitions with CaV2.1into lipid rafts in cerebellum: implications for localization and function. J Neurosci. August 23; 26(34):8748-57.

Dawes, C. 1987. Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. J Dent Res. Feb;66 Spec No:648-53

Dawes C. 1993. Considerations in the development of diagnostic tests on saliva. Ann N Y Acad Sci. Sep 20; 694:265-9.

Dawes, C. 2004. How much saliva is enough for avoidance of xerostomia? Caries Res. May-Jun; 38(3):236-40. Review.

Dawes, C.2008.Salivary flow patterns and the health of hard and soft oral tissues. J Am Dent Assoc. May; 139 Suppl; 18S-24S.Review.

Dawes, C., Dibdin G., H. 2001. Salivary concentrations of urea released from a chewing gum containing urea and how these affect the urea content of gel-stabilized plaques and their pH after exposure to sucrose. Caries Res. Sep-Oct; 35(5):344-53.

De Bruyn, R., S., Stern R., O. 1929. A case of the progressive hypertrophic polyneuritis of Dejerine and Sottas, with pathological examination. Brain; 52(3): 84-107.

De Jong, M., H., Van der Hoeven J., S. 1987. The growth of oral bacteria on saliva. J Dent. Res. Feb; 66(2): 498-505.

Delahaye, N., Dinanian S., Slama M., S., Mzabi H., Samuel D., Adams D.,Merlet P., Le Guludec D.1999. Cardiac sympathetic denervation in familial amyloid polyneuropathy assessed by iodine-123 metaiodobenzylguanidine scintigraphy and heart rate variability. Eur J Nucl Med. Apr; 26(4):416-24.

Delgado, W., A., Mosqueda A. 1989. A highly sensitive method for diagnosis of secondary amyloidosis by labial salivary gland biopsy. J Oral Pathol Med. May; 18(5):310-4.

Delgado, W., A., Arana-Chavez V., E. 1997.Amyloid deposits in labial salivary glands identified by electron microscopy. J Oral Pathol Med. Jan; 26(1):51-2.

Dibdin G., Dawes C. 1998. A mathematical model of the influence of salivary urea on the pH of fasted dental plaque and on the changes occurring during a cariogenic challenge. Caries Res. 32(1):70-4.

Di Gioia, M., L., Leggio A., Le Pera A., Liguori A., Napoli A., Siciliano C., Sindona G. 2004. Quantitative analysis of human salivary glucose by gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. Mar 5; 801(2):355-8.

Dickinson, W., H. 1869. Lardaceous disease of the kidney consequent upon abscess of the ovary. Trans Path Soc Lond. 20:435-9.

Dickson, P.,W, Aldred A.,R., Marley P.,D., Tu G,F., Howlett G.,J., Schreiber G. 1985. High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain. Biochem Biophys Res Commun. Mar 29; 127(3):890-5.

Díez-Itza, I., Sánchez L., M., Allende M., T., Vizoso F., Ruibal A., López-Otín C. 1993. Zn-alpha 2-glycoprotein levels in breast cancer cytosols and correlation with clinical, histological and biochemical parameters. Eur J Cancer. 29A (9):1256-60.

Ding, M., Umetsu K., Nakayashiki N., Choi W., Y., Jia., Suzuki T. 1990. Distribution of human Zn-alpha-2-glycoprotein types in Chinese and Korean populations. Hum Hered. 40(5):311-2.

Ding, Q., Keller J., N. 2005.Evaluation of rage isoforms, ligands, and signaling in the brain. Biochim Biophys Acta. Oct 30; 1746 (1):18 – 27. Review.

Divry, P., Florkin M. 1927. Sur les propriétés optiques de l'amylöide. CR Soc Biol. 97: 1808-1810.

Do Amaral, B., Coelho T., Sousa A, Guimarães A. 2009U.sefulness of labial salivary gland biopsy in familial amyloid polyneuropathy Portuguese type. Amyloid. Dec; 16(4): 232-8.

Dodds, M., J., Yeh C., K., Johnson D., A. 2000. Salivary alterations in type 2 (non-insulin dependent) diabetes mellitus and hypertension. Community Dent Oral Epidemiol. Oct; 28(5):373–81.

Dodds, M., W., Johnson D., A., Yeh C., K.2005. Health benefits of saliva: a review. J Dent. Mar; 33(3): 223-33.

Doyle L.1988.Lardaceous disease: some early reports by British authors (1722-1879). J R Soc Med. Dec; 81(12):729-31.

Drobni, M., Li T., Kruger C., Loimaranta V., Kilian M., Hammarström L., Jörnvall H., Bergman T., Strömberg N. 2006. Host-derived pentapeptide affecting adhesion, proliferation, and local pH in biofilm communities composed of Streptococcus and Actinomyces species. Infect Immun. Nov; 74(11):6293-9.

Dubois, V., Delort L., Mishellany F., Jarde T., Billard H., Lequeux C., Damour O., Penault-Llorca F., Vasson M.,P., Caldefie-Chezet F. 2010. Zinc-alpha2-glycoprotein: a new biomarker of breast cancer? Anticancer Res. Jul; 30(7): 2919-25.

Dubový, P., Klusáková I., Svizénská I.2002.A quantitative immunohistochemical study of the endoneurium in the rat dorsal and ventral spinal roots. Histochem Cell Biol. Jun; 117(6): 473-80.

Ducla-Soares, J., Alves M., M., Carvalho M., Póvoa P., Conceição I., Sales Luis M., L. 1994.Correlation between clinical, electromyographic and dysautonomic evolution of familial amyloid polyneuropathy of the Portuguese type. Acta Neurol Scand. Oct; 90(4):266-9.

Ducloux, D. 2011. Physiopathologie et traitement des complications du syndrome néphrotique. Néphrol Ther. Feb; 7(1):52-7.

Dyck, P.J., Lambert, G.H., 1969. Dissociated sensation in amyloidosis. Compound action potential quantitative histologic and teased-fiber and electron microscopic studies of sural nerve biopsies. Arch Neurol. May; 20(5):490–507

Eanes, E., D., Glenner, G., G. 1968. X-ray diffraction studies on amyloid filaments. J Histochem Cytochem. Nov; 16(11):673-7.

Edgerton, M., Scannapieco F., A., Reddy M., S., Levine M., J. 1993.Human submandibular-sublingual saliva promotes adhesion of Candida albicans to polymethylmethacrylate. Jun; Infect Immun. June; 61(6):2644-52.

Emmelin, N.1987. Nerve interactions in salivary glands. J Dent Res. Feb; 66(2):509-17.

Engelen, L., van den Keybus P., A.,M., de Wijk R.,A., Veerman E.,C., Amerongen A.,V., Bosman F., Prinz J., F., van der Bilt A. 2007.The effect of saliva composition on texture perception of semi-solids. Arc Oral Biol. Jun; 52(6):518-25.

Espin-Ferra, J., Merida-Velasco J.,A., Garcia-Garcia J.,D, Sanchez-Montesinos I., Barranco-Zafra R., J.1991.Relationships between the parotid gland and the facial nerve during human development. J Dent Res. Jul; 70(7):1035-40.

Fábián, T., K., Fejérdy P., Nguyen M., T., Soti C., Csermely P. 2007.Potential immunological functions of salivary Hsp70 in mucosal and periodontal defense mechanisms. Arch Immunol Ther Exp (Warsz).Mar-Apr; 55(2):91-98. Review.

Falk, R., H., Comenzo R., L, Skinner M. 1997. The systemic amyloidoses. N Engl J Med. Sep 25; 337(13):898-909.

Fenoll-Palomares, C., Muñoz Montagud J., V., Sanchiz V., Herreros B., Hernández V., Mínguez M., Benages A. 2004.Unstimulated salivary flow rate, pH and buffer capacity of saliva in healthy volunteers. Rev Esp Enferm Dig. Nov; 96(11):773-83.

Ferguson, D., B.1987.Current diagnostic uses of saliva. J Dent Res. Feb; 66(2): 420-4. Review.

Ferguson, D., B. 1999.The flow rate and composition of human labial gland saliva Arch Oral Biol. May; 44 Suppl 1:S11-14. Review.

Ferrão-Gonzales, A., D., Palmieri L., Valory M., Silva J., L., Lashuel H., Kelly J., W., Foguel D. 2003. Hydration and packing are crucial to amyloidogenesis as revealed by pressure studies on transthyretin variants that either protect or worsen amyloid disease. J Mol Biol. May 9; 328(4): 963-74.

Fisher, S., Z., Govindasamy L., Tu C., Agbandje-McKenna M., Silverman D., N., Rajaniemi H., J., McKenna R.2006. Structure of human salivary alpha-amylase crystallized in a C-centered monoclinic space group. Acta Crystallogr Sect F Struct Biol Cryst Commun. Feb 1; 62(Pt 2):88-93.

Flink, H., Tegelberg A., Sörensen S. 2000. Hyposalivation and iron stores among individuals with and without active dental caries. Acta Odontol Scand. Dec; 58(6):265-71.

Fonseca, C., Ceia F., Carvalho A., Nogueira J., S., Morais H., Conceição I., Luis M., L., Luis A., S., 1997. História natural do envolvimento cardíaco na polineuropatia amiloidótica familiar do tipo português. Rev Port Cardiol. Jan; 16(1):101-5.

Freel, S.,A., Williams J., M., Nelson J., A., E., Patton L., L., Fiscus S., A., Swanstrom R, Shugars D., C. 2001. Characterization of human immunodeficiency virus type 1 in saliva and blood plasma by V3-specific heteroduplex tracking assay and genotype analyses. J Virol. May; 75(10):4936-40.

Freije, J., P., Fueyo A, Uría J., López-Otín C. 1991. Human Zn-alpha 2-glycoprotein cDNA cloning and expression analysis in benign and malignant breast tissues. FEBS Lett. Sep 23; 290(1-2):247-9.

Freitas, A., F.1986.The heart in Portuguese amyloidosis. Postgrad Med J. Jun; 62(728):601-601.

Frenette, G., Dubé J.,Y., Lazure C., Paradis G., Chrétien M., Tremblay R.,R. 1987. The major 40-kDa glycoprotein in human prostatic fluid is identical to Zn-alpha 2-glycoprotein.Prostate.11 (3): 257-70

Gabriel, C.,M., Howard R., Kinsella N., Lucas S., McColl I., Saldanha G., Hall S.,M., Hughes R. 2000. A Prospective study of the usefulness of sural nerve biopsy. J Neurol Neurosurg Psychiatry. Oct; 69(4):442-6.

Gallo, G., Wisniewski T., Choi-Miura N., H., Ghiso J., Frangione B. 1994. Potential role of apolipoprotein-E in fibrillogenesis. Am J Pathol. Sep; 145(3):526-30.

Gannot, G., Lancaster H., E., Fox P., C. 2000. Clinical course of primary Sjögren's syndrome: salivary, oral, and serologic aspects. J Rheumatol. Aug; 27(8):1905-9.

Gendler, S., J., Spicer A., P. 1995. Epithelial mucin genes. Annu Rev Physiol.57:607-34.Review.

Gertz, M., A, Lacy M., Q., Dispenzieri A., Hayman S., R. 2005. Amyloidosis: diagnosis and management. Clin Lymphoma Myeloma. Nov; 6(3):208-19.

Glenner G.,G., Ein D., Eanes E.,D., Bladen H.,A., Terry W., Page D..L.1971b. Creation of “amyloid” fibrils from Bence Jones protein in vitro. Science. Nov; 174(10):712-4.

Ghezzi, E., .M. Lange L.., A., Ship J., A.2000.Determination of variation of stimulated salivary flow rates. J Dent Res. Nov; 79(11):1874-8.

Gorr, S., U., Venkatesh S., G., Darling D., S. 2005.Parotid Secretory Granules: Crossroads of Secretory Pathways and Protein Storage. J Dent Res. Jun; 84(6):500-509.

Goicovich, E., Molina C., Pérez P., Aguilera S., Fernández J., Olea N., Alliende C., Leyton C., Romo R., Leyton L., González M., J. 2003. Enhanced degradation of proteins of the basal lamina and stroma by matrix metalloproteinases from the salivary glands of Sjögren's syndrome patients: correlation with reduced structural integrity of acini and ducts. Arthritis Rheum. Sep; 48(9):2573-84.

Golub, L., M., Borden S., M., Kleinberg I. 1971. Urea content of gingival crevicular fluid and its relation to periodontal diseases in humans. J Periodontal Res. 684):243-51.

Gorlin, R., Gottsegen R. 1949. The role of the gingival biopsy in secondary amyloid disease. Oral Surg Oral Med Oral Pathol. Jul; 2(7):864-6.

Guimarães, A., Monteiro L., Coutinho P. Pathology of the autonomic nervous system in andrade type of familial amyloidotic polyneuropathy. In: Glenner, G.G., Costa, P.P., de Freitas, A.F. (Eds.).Amyloid and Amyloidosis. Excerpta Medica, Amsterdam, 1980.pp. 88-98.

Guimarães, A., Pinheiro A., V., Leite I. Sural nerve biopsy in familial amyloidotic polyneuropathy: a morphological and morphometric polyneuropathy. In: Isobe T., Araki A., Uchino F., Kito S., Tsubura E., editors. Amyloid and amyloidosis. New York: Plenum Press; 1988. pp 493-498.

Habte, H., H, Mall A., S., de Beer C., Lotz Z., E., Kahn D. 2006. The role of crude human saliva and purified salivary MUC5B and MUC7 mucins in the inhibition of Human Immunodeficiency Virus type 1 in an inhibition assay. Virol J. Nov 24; 3:99.

Hachulla, E., Janin A., Flipo R.,M., Saïle R., Facon T., Bataille D., Vanhille P., Hatron P.,Y., Devulder B., Duquesnoy B. 1993. Labial salivary gland biopsy is a reliable test for the diagnosis of primary and secondary amyloidosis. A prospective clinical and immunohistologic study in 59 patients. Arthritis Rheum. May; 36(5):691-7.

Hakeem, V., Fifield R., Al-Bayaty H., F., Aldred M., J., Walker D., M., Williams J., Jenkins H., R.1992.Salivary IgA antigliadin antibody as a marker for coeliac disease. Arch Dis Child. Jun; 67(6):724-7.

Hansen, T., V., O., Simonsen M., K., Nielsen F., C., Hundrup Y., A.2007.Collection of blood, saliva, and buccal cell samples in a pilot study on the Danish nurse cohort: comparison of the response rate and quality of genomic DNA. Cancer Epidemiol Biomarkers Prev. Oct; 16(10):2072-6.

Hardt, M., Thomas L.,R., Dixon S.,E., Newport G., Agabian N., Prakobphol A., Hall S.,C., Witkowska H.E.,, Fisher S.,J. 2005. Toward defining the human parotid gland

salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry. Mar 1; 44(8):2885-99.

Hawkins, P., N., Lavender, J., P., Pepys, M., B. 1990. Evaluation of systemic amyloidosis by scintigraphy with 233I-labeled serum amyloid P component. N Engl J Med.323:508-13.

Henskens, Y., M., van der Velden U., Veerman E., C., Nieuw Amerongen A., V. 1993. Protein, albumin and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis or periodontitis. J Periodontal Res. Jan; 28(1):43-8.

Henshall, S.,M., Horvath L.,G., Quinn D.,I., Eggleton S.,A., Grygiel J.,J., Stricker P.,D., Biankin A.,V., Kench J.,G., Sutherland R.,L. 2006. Zinc-alpha2-glycoprotein expression as a predictor of metastatic prostate cancer following radical prostatectomy. J Natl Cancer Inst. Oct 4; 98(19):1420-4.

Hibbs, M.,S., Hasty K.,A., Seyer J.,M., Kang A.,H., Mainardi C.,L.1985. Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. J Biol Chem. Feb 25; 260(4):2493-500.

Hirai, K., Hussey H., J., Barber M., D., Price S., A., Tisdale M., J. 1998. Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients. Cancer Res. Jun 1; 58(11):2359-65.

Hodson, N., A. Linden R., W.2006.The effect of monosodium glutamate on parotid salivary flow in comparison to the response to representatives of the other four basic tastes. Physiol Behav. Dec 30; 89(5):711-7.

Hofer, P., A., Andersson R.1975.Postmortem findings in primary familial amyloidosis with polyneuropathy: a study based on six cases from northern Sweden. Acta Path Microbiol Scand A. May; 83(3):309-22.

Hou, X., Richardson S., J., Aguilar M., I., Small D., H.2005.Binding of amyloidogenic transthyretin to the plasma membrane alters membrane fluidity and induces neurotoxicity. Biochemistry. Aug; 30; 44(34):11618-27.

Hou, X., Parkington H., C., Coleman H., A., Mechler A., Martin L., L., Aguilar M., I., Small D., H. 2007. Transthyretin oligomers induce calcium influx via voltage-gated calcium channels. J Neurochem. Jan; 100(2):446-57.

Hsu, J., C., Yamada K., M 2010., Salivary Gland Branching Morphogenesis- Recent Progress and Future Opportunities. Int J Oral Sci. 2(3):117-26.

Hu, S., Loo J., A., Wong D., T. 2007. Human saliva proteome analysis and disease biomarker discovery. Expert Rev Proteomics. Aug; 4(4):531-38.Review.

Hu, S., Wang J., Meijer J., Ieong S., Xie Y. , Yu T., Zhou H., Henry S., Vissink A., Pijpe J., Kallenberg C., Elashoff D., Loo j., A., Wong D.,T. 2007. Salivary proteomic and genomic biomarkers for primary Sjögren`s syndrome. Arthritis Rheum. Nov; 56(11): 3588-600.

Humphrey, S., P., Williamson R., T., 2001. A review of saliva: normal composition, flow, and function. J Prosthet Dent. Feb; 85(2):162-9.

Husby G., Stenstad T., Magnus J., H., Sletten K., Nordväg B., Y., Marhaug G.1994. Interaction between circulating amyloid fibril protein precursors and extracellular tissue matrix components in the pathogenesis of systemic amyloidosis. Clin Immunol Immunopathol. Jan; 70 (1):2-9.

I., Aviv., S., Avraham., M., Koren-Michowitz., T., Zuckerman., A. Aviv., Y., Ofran., N., Benyamini., A., Nagler., J.,M., Rowe., R.,M Nagler. 2009. Oral integrity and salivary profile in myeloma patients undergoing high-dose therapy followed by autologous SCT. Bone Marrow Transplantation. May; 43(10); 801-6.

Ibrahim, H., R., Matsuzaki .T, Aoki T. 2001. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett. Sep 28; 506(1):27-32.

Ihalin, R., Loimaranta V., Tenovuo J.2006.Origin, structure, and biological activities of peroxidases in human saliva. Arch Biochem Biophys. Jan: 15; 445(2):261-8.Review.

Ikeda S., I., Oguchi K., Yanagisawa N., Nagata T. 1982. Gastrointestinal amyloid deposition in familial amyloid polyneuropathy. Neurology. Dec; 32(12):1364-8.

Ikeda, S., Nakazato M., Ando Y., Sobue G. 2002.Familial transthyretin-type amyloid polyneuropathy in Japan clinical and genetic heterogeneity. Neurology. Apr 9; 58(7):1001-7.Review.

Inoue, S., Kuroiwa M., Saraiva M., J. Guimarães A., Kisilevsky R.1998. Ultrastructure of familial amyloid polyneuropathy amyloid fibrils: examination with high-resolution electron microscopy. J Struct Biol. Dec 1; 124(1):1-12.

J. Pereira Guedes.1976. Boletim do Hospital. 1(1): 51-59.

Jain, S., Rajput A., Kumar Y., Uppuluri N., Arvind A., S., Tatu U. 2005. Proteomic analysis of urinary protein markers for accurate prediction of diabetic kidney disorder. J Assoc Physicians India. Jun; 53:513-20.

Jang, W., S, Bajwa J., S., Sun J., N., Edgerton M.2010.Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in Candida albicans. Molecular Microbiology. 77:354-70.

Johansson, I. , Ryberg M., Steen L., Wigren L. 1992. Salivary hypofunction in patients with familial amyloidotic polyneuropathy. Oral Surg Oral Med Oral Pathol. Dec; 74(6):742-8.

Juusela P., Tanskanen M., Nieminen A., Uitto V.,J., Blåfield H., Kiuru-Enari S. 2009. Hereditary gelsolin amyloidosis mimicking Sjögren's syndrome. Clin Rheumatol. Nov; 28(11):1351-4.

Kalk, W.,W., Vissink A., Stegenga B., Bootsma H., Nieuw Amerongen A.,V., Kallenberg C.,G. 2002. Sialometry and sialochemistry: a non-invasive approach for diagnosing Sjögren's syndrome. Ann Rheum Dis. Feb; 61(2):137-44

Kanda, Y., Goodman D., S., Canfield R., E., Morgan F., J. 1974. The amino acid sequence of human plasma prealbumin. J Biol Chem. Nov 10; 249(21):6796-805.

Kang, J., G., Kim S., H., Ahn T., Y. 2006. Bacterial diversity in the human saliva from different ages. J. Microbiol. Oct; 44(5):572-6.

Kariyawasam, A., P., Dawes C. 2005. A circannual rhythm in unstimulated salivary flow rate when the ambient temperature varies by only about 2 degrees C. Arch Oral Biol. Oct;50(10):919-22.

Keith, D., A. 1972. Oral features of primary amyloidosis. Br J Oral Surg. Nov; 10(2): 107-15.

Kelly, J., W. 1997. Amyloid fibril formation and protein misassembly: a structural quest for insights into amyloid and prion diseases. Structure. May 15; (5):595-600.

Kelly, S., M., Jess T., J., Prince N.,C. 2005. How to study proteins by circular dichroism. Biochim Biophys Acta 1751: 119-139.

Khader, Y., S., T, Dauod A., S., El-Qaderi S., S., Alkafajei A., Batayha W., Q.2006. Periodontal status of diabetics compared with nondiabetics: a meta-analysis. J Diabetes Complications. Jan-Feb; 20(1):59-68.

Khoury, S., Dusek J., J., Anderson G., B., Vigneswaran N. 2004.Systemic amyloidosis manifesting as localized, severe periodontitis. J Am Dent Assoc. May; 135(5):617-23.

Kopstein, J., Wrong O., M. 1997. The origin and fate of salivary urea and ammonia in man. Clin Sci Mol Med. Jan; 52(1)9-17

Konttinen Y.,T., Halinen S., Hanemaaijer R., Sorsa T., Hietanen J., Ceponis A., Xu J.,W., Manthorpe R., Whittington J., Larsson A., Salo T., Kjeldsen L., Stenman U.,H., Eisen A.,Z. 1998. Matrix metalloproteinase (MMP)-9 type IV collagenase/gelatinase implicated in the pathogenesis of Sjögren's syndrome. Matrix Biol. Oct; 17(5):335-47.

Kumar, S., V. Hurteau G., J., Spivack S., D.2006.Validity of messenger RNA expression analyses of human saliva. Clin Cancer Res. Sep 1; 12(17):5033-9.

Lai, Z., Colón W., Kelly J., W.1996.The acid-mediated denaturation pathway of transthyretin yields a conformational intermediate that can self-assemble into amyloid. Biochemistry.May21; 35(20):6470-82.

Lakowicz, J.R. 1987. Principles of fluorescence spectroscopy. Plenum Press, New York and London.

Lambert, M., P., Barlow A., K., Chromy B., A., Edwards C., Freed R., Liosatos M., Morgan T., E., Rozovsky I., Trommer B., Viola K., L., Wals P., Zhang C., Finch C., E., Krafft G., A., Klein W., L.1998.Diffusible, nonfibrillar ligands derived from Abeta1–42 are potent central nervous system neurotoxins. Proc Natl Acad Sci USA. May 26; 95(11) 6448-53.

Lashuel, HA, Lai Z, Kelly JW. 1998 Characterization of the transthyretin acid denaturation pathways by analytical ultracentrifugation: implications for wild-type, V30M, and L55P amyloid fibril formation. Biochemistry. Dec 22; 37(51):17851-64.

Lebowitz, R., A., Morris L. 2003. Plasma cell dyscrasias and amyloidosis. Otolaryngol Clin North Am. Aug; 36(4):747-64.

Lechapt-Zalcman, E., Authier F., J., Creange A., Voisin M., C., Gherardi R.,K. 1999. Labial salivary gland biopsy for diagnosis of amyloid polyneuropathy. Muscle Nerve. Jan; 22(1):105-7.

Lee J., Y., Kim Y., Y., Chang J., Y., Park M., S., Kho H., S. 2010.The effects of peroxidase on the enzymatic and candidacidal activities of lysozyme. Arch Oral Biol. Sep; 55(9):607-12.

Lei, G., Arany I., Selvanayagam P., Rajaraman S., Ram S., Brysk H., Tyring S.,K., Brysk M.,M. 1997. Detection and cloning of epidermal zinc-alpha 2-glycoprotein cDNA and expression in normal human skin and in tumors. J Cell Biochem. Nov 1; 67(2):216-22.

Lei, G., Brysk H., Arany. Tyring. Srinivasan G., Brysk M., M. 1999. Characterization of zinc-alpha (2)-glycoprotein as a cell adhesion molecule that inhibits the proliferation of an oral tumor cell line. J Cell Biochem. Oct 1; 75(1):160-9.

Lei, M., Mingfeng Y., Shuanghong H.2004. Intrinsic versus mutation dependent instability/flexibility: a comparative analysis of the structure and dynamics of wild-type transthyretin and its pathogenic variants. J Struct Biol. Nov; 148(2):153-68.

Leite, I., Coutinho P., Viana Pinheiro A., Guimarães A., Saraiva M.J., Costa P.P., 1988. Familial amyloid polyneuropathy (Portuguese type): Study of asymptomatic carriers. In: Isobe, T., Araki S., Uchino F., Kito S., Tsubura E. (Eds.), Amyloid and Amyloidosis. Plenum Press, New York, pp. 429-434.

Lemos, J., A., Chen Y., Y., M., Burne R., A. 2001.Genetic and physiologic analysis of the groE operon and role of the HrcA repressor in stress gene regulation and acid tolerance in Streptococcus mutans. J. Bacteriol. Oct; 183(20):6074-84.

Lenander-Lumikari, Johansson I., Vilja P., Samaranayake L., P. 1995. Newer saliva collection methods and saliva composition: a study of two Salivette kits. Oral Dis. Jun; 1(2):86-91

Li, S., Sommer S., S. 1993. The high frequency of TTR M^{30} in familial amyloidotic polyneuropathy is not due to a founder effect. Hum Mol Genet. 2: 1303-1305.

Li, Y., Ku C.Y., Xu, J., Saxena D., Caufield P., W. 2005. Survey of oral microbial diversity using PCR-based denaturing gradient gel electrophoresis. J Dent Res. Jun; 84(6):559-64.

Li, Y., St John M., A., Zhou X., kim Y., Sinha U., Jordan R., C., Eisele D., Abemayor E., Elashoff D., Park N., H., Wong D., T. 2004.Salivary transcriptome diagnostics for oral cancer detection. Clin Cancer Res. Dec 15; 10(24):8442-50.

Libbey C., A, Skinner M., Cohen A., S. 1983. Use of abdominal fat tissue aspirate in the diagnosis of systemic amyloidosis. Arch Intern Med. Aug; 143(8):1549-52

Lighterman, I. 1951. A modified technique for gingival biopsy in the diagnosis of secondary amyloidosis. Oral Surg Oral Med Oral Pathol. Mar; 4(3):351-4.

Lin, A., L., Johnson D.A., Stephan K.T., Yeh C.K.2003.Alteration in salivary function in early HIV infection. J Dent Res. Sep; 82(9):719-24.

Liu, D., Niemann M., Hu K., Herrmann S., Störk S., Knop S., Ertl G., Weidemann F. 2011. Echocardiographic evaluation of systolic and diastolic function in patients with cardiac amyloidosis. Am J Cardiol. Aug 15; 108(4):591-8.

Lobato, L., Beirão I., Silva M., Bravo F., Silvestre F., Guimarães S., Sousa A., Noël L.H., Sequeiros J.2003.Familial ATTR amyloidosis: microalbuminuria as a predictor of symptomatic disease and clinical nephropathy. Nephrol Dial Transplant. Mar; 18(3):532-8.

Loimaranta, V., Tenovuo J., Koivisto L., Karp M.1998.Generation of bioluminescent Streptococcus mutans and Its usage in rapid analysis of the efficacy of antimicrobial compounds. Antimicrob Agents Chemother. Aug; 42(8):1906-10.

Lopez-Jornet, M., P., Bermejo-Fenoll 1994. A. Is there na age-dependent decrease in resting secretion of saliva of healthy persons? A study of 1493 subjects. Braz Dent J.5 (2):93-8.

Lovett, D., W. Cross K., R., Van Allen M. 1965. The prevalence of amyloids in gingival tissues. Oral Surg Oral Med Oral Pathol. Oct; 20(4):444-8.

Luis, M., L.1978. Electroneurophysiological studies in familial amyloid polyneuropathy-Portuguese type. J Neurol Neurosurg Psychiatry. Sep; 41(9):847-50.

Luyendijk, W., Bots G.T., Vegter-van der Vlis M., Went L., N., Frangione B.1988. Hereditary cerebral haemorrhage caused by cortical amyloid angiopathy. J Neurol Sci. Jul; 85(3):267-80.

Lyon, A.,W., Narindrasorasak S., Young I.,D., Anastassiades T., Couchman J.,R., McCarthy K., J., Kisilevsky R. 1991. Co-deposition of basement membrane components during the induction of murine splenic AA amyloid. Lab Invest. Jun; 64(6):785-90.

Macedo, B., Batista A., R, do Amaral J., B., Saraiva M., J. 2007. Biomarkers in the assessment of therapies for familial amyloidotic polyneuropathy. Mol Med. Nov-Dec; 13(11-12):584-91.

Macedo, E., Coutinho P., Morais R., Matos E., 1988. Familial amyloid polyneuropathy: a clinical scale for the evaluation of the peripheral neuropathy. In Isobe T., Araky A., Uchino F., Kito S., Tsubura E. (Eds). Amyloid and Amyloidosis. Plenum Press, New York, 1988:pp419-423.

Magnus-Levy, A.1931.Bence-Jones-Eiweiss und amyloid. Zeitschrift für Klinische Medizinische, 116, 510-531.

Mainardi, C., L., Hibbs M., S., Hasty K., A., Seyer J., M. 1984. Purification of a type V collagen degrading metalloproteinase from rabbit alveolar macrophages. Coll Relat Res. Dec; 4(6):479-92.

Mäkelä, M., Salo T., Uitto V., J., Larjava H. 1994.status Dent Res. Aug; 73(8):1397-406.

Malamud, D.1992.Saliva as a diagnostic fluid. BMJ. Jul 25; (6847):207-8.

Mandel, I., D.1980. Sialochemistry in diseases and clinical situations affecting salivary glands. Crit Rev Clin Lab Sci.12(4):321-66.

Mandel, I., D. 1993. Salivary diagnosis: promises, promises. Ann N Y Acad Sci. Sep 20; 694:1-10.

Mann, M., and Wilm M. 1995. Electrospray mass spectrometry for protein characterization. Trends Biochem. Sci. 20: 219-224

Mardinger, O., Rotenberg L., Chaushu G., Taicher S. 1999. Surgical management of macroglossia due to primary amyloidosis. Int J Oral Maxillofac Surg. Apr; 28(2):129-31.

Mathews, S., A., Kurien B., T., Scofield R., H. 2008. Oral manifestations of Sjögren`s syndrome. J Dent Res. Apr; 87(4):308-18.

Matsunaga, N., Anan I., Forsgren S., Nagai R., Rosenberg P., Horiuchi S., Ando Y., Suhr O.,B. 2002. Advanced glycation end products (AGE) and the receptor for AGE are present in gastrointestinal tract of familial amyloidotic polyneuropathy but do not induce NF-kB activation. Acta Neuropathol. Nov; 104(5):441-7.

Matsubara, K., Mizuguchi M., Igarashi K., Shinohara Y., Takeuchi M., Matsuura.A., Saitoh T., Mori Y., Shinoda H., Kawano K.2005.Dimeric transthyretin variant assembles into spherical neurotoxins. Biochemistry**.** Mar 8; 44(9):3280-8.

Mazzeo, M., A., Linares J.,A., Campos M.,L., Busamia B., E., Dubersarsky C., Lavarda M., Jarchum G., Finkelberg A., B. 2009.Oral signs of intravenous chemotherapy with 5- Fluorouracil and Leucovorin calcium in colon cancer treatment. Med Oral Patol Oral Cir Bucal. Mar 1; 14 (3): E108-13.

Mellanen, L., Sorsa T., Lähdevirta J., Helenius M., Kari K., Meurman J., H.2001. Salivary albumin, total protein, IgA, IgG and IgM concentrations and occurrence of some periodontopathogens in HIV-infected patients: a 2-year follow-up study. J Oral Pathol Med. Oct; 30(9):553-9.

Merlini, G., Stone M., J.2006. Dangerous small B-cell clones. Blood. Oct 15; 10(8):2520-30.Review.

Meurman J., H., Grönroos L, 2010. Oral and dental health care of oral cancer patients: hyposalivation, caries and infections Oral Oncol. Jun; 46(6):464-7.

Millard, H., D., Mason D., K. editors. Third World Workshop on Oral Medicine. Ann Arbor (MI): University of Michigan: 1998

Michishige, F., Kanno K., Yoshinaga S., Hinode D., Takehisa Y., Yasuoka S. 2006. Effect of saliva collection method on the concentration of protein components in saliva. J Med Invest. Feb; 53(1-2):140-6.

Misu, K., Hattori N., Nagamatsu M., Ikeda S., Ando Y., Nakazato M., Takei Y., Hanyu N., Usui Y., Tanaka F., Harada T., Inukai A., Hashizume Y., Sobue G. 1999. Late-onset familial amyloid polyneuropathy type I (transthyretin Met30-associated familial amyloid polyneuropathy) unrelated to endemic focus in Japan. Clinicopathological and genetic features. Brain. Oct; 122(Pt 10):1951-62.

Mizukawa, N., Sugiyama K., Fukunaga J., Ueno T., Mishima K., Takagi S., Sugahara T.1998.Defensin-1, a peptide detected in the saliva of oral squamous cell carcinoma patients. Anticancer Res. Nov-Dec; 18(6B):4645-9.

Monteiro, F. A., Sousa M., M., Cardoso I., do Amaral J., B., Guimarães A., Saraiva M.,J.2006.Activation of ERK1/2 MAP kinases in familial amyloidotic polyneuropathy. J Neurochem. Apr; 97(1):151-61.

Moore, P., A, Guggenheimer J.2008.Medication-induced hyposalivation: etiology, diagnosis and treatment. Compend Contin Educ Dent. Jan-Feb; 29(1):50-5 Review.

Munar-Qués, M., Saraiva M., J., Viader-Farré C., Zabay-Becerril J., M., Mulet-Ferrer J. 2005. Genetic epidemiology of familial amyloid polyneuropathy in the Balearic Islands (Spain). Amyloid. Mar; 12(1): 54-6.Review.

Nagler, R., M., Nagler A. 2003. Sialometrical and sialochemical analysis of patients with chronic graft-versus-host disease- a prolonged study. Cancer Invest.21 (1):34-40.

Nandan, R., K., Sivapathasundharam B., Sivakumar G. 2005. Oral manifestations and analysis of salivary and blood urea levels of patients under going haemo dialysis and kidney transplant. Indian J Dent Res. Jul-Sep; 16(3):77-82.

Nandapalan, V., Jones T., M., Morar P., Clark A., H., Jones A., S. 1998. Localized amyloidosis of the parotid gland: a case report and review of the localized amyloidosis of the head and neck. Head Neck. Jan; 20(1):73-8.

Nascimento M., M., Gordan V., V., Garvan C., W., Browngardt C., M., Burne R.,A. 2009.Correlations of oral bacterial arginine and urea catabolism with caries experience. Oral Microbiol Immunol. Apr; 24(2):89-5.

Nater, U., M., Rohleder N. 2009. Salivary alpha-amylase as a non invasive biomarker for the sympathetic nervous system: current state of research. Psychoneuroendocrinology. May; 34(4):486-96. Review.

Navazesh, M., Christensen C., Brightman V. 1992. Clinical criteria for the diagnosis of salivary gland hypofunction. J Dent Res. Jul; 71(7):1363-9.

Navazesh, M. 1993. Methods for collecting saliva. Ann N Y Acad Sci. Sep 20; 694:72-7.

Navazesh, M. 2003. How can oral healthcare providers determine if patients have dry mouth? J Am Dent Assoc. May; 134(5):613-20.

Navazesh, M Kumar S., K. 2008. Measuring salivary flow: Challenges and opportunities. J Am Dent Assoc. May; 139 Suppl: 35S-40S.

Nederfors, T. 2000.Xerostomia and hyposalivation. Adv Dent Res. Dec; 14:48-56.

Noble, R., E. 2000. Salivary alpha-amylase and lysozyme levels: a non-invasive technique for measuring parotid vs submandibular/sublingual gland activity. J Oral Sci. Jun; 42(2):83-6.

Nogueira, R.,D., Alves A., C., Napimoga M.,H., Smith D.,J., Mattos-Graner R.,O. 2005.Characterization of salivary immunoglobulin A responses in children heavily exposed to the oral bacterium Streptococcus mutans: influence of specific antigen recognition in infection. Infect Immun. Sept; 73/9):5675-84.

Nordlie, M., Sletten K., Husby G., RanlØv P., J. 1988. A new prealbumin variant in familial amyloid cardiomyopathy of Danish origin. Scand J Immunol. Jan; 27(1):119-22.

O'Connell, K., M., Martens J., R., Tamkun M., M. 2004.Localization of ion channels to lipid raft domains within the cardiovascular system. Trends Cardiovasc Med. Feb; 14(2):37-42.

Offner, G., D, Troxler R., F. 2000. Heterogeneity of high-molecular-weight human salivary mucins. Adv Dent Res. Dec; 14: 69-75.

Ogami, K., Sakurai K., Ando T. 2004.A method of measuring salivary flow rate in the lower labial mucosal region. J Oral Rehabil. Sep; 31(9):861-5.

Ohkubo, I., Niwa M., Takashima A., Nishikimi N., Gasa S., Sasaki M. 1990. Human seminal plasma Zn-alpha 2-glycoprotein: its purification and properties as compared with human plasma Zn-alpha 2-glycoprotein. Biochim Biophys Acta. May 16; 1034(2):152-6.

Olafsson, I., Grubb A.2000.Hereditary cystatin C amyloid angiopathy. Amyloid: Mar; 7(1):70-9.

Ostertag, B.1950. Familiare amyloid-erkrankung. Ztschr. menschl. Vererb. u. Konstitutionslehre 30:105-115.

Palácios, S.,A,, Bittencourt P.,L., Cançado E.,L., Farias A.,Q., Massarollo P.,C., Mies S., Kalil J., Goldberg A.,C.1999.Familial amyloidotic polyneuropathy type 1 in Brazil is associated with the transthyretin Val30Met variant. Amyloid. Dec; 6(4):289-91.

Paccalin, M., Hachulla E., Cazalet C., Tricot L., Carreiro M., Rubi M., Grateau G., Roblot P.2005.Localized amyloidosis: A survey of 35 French cases. Amyloid, Dec; 12(4):239-245.

Park, N., J., Li Y., Yu T., Brinkman B., M., Wong D., T. 2006. Characterization of RNA in saliva. Clin Chem. Jun; 52(6):988-94.

Park, N., J., Zhou X., Yu T., Brinkman B., M., Zimmermann B., G., Palanisamy V., Wong D.,T. 2007. Characterization of salivary RNA by cDNA library analysis. Arch Oral Biol. Jan; 52(1):30-5.

Pedersen, A., M., Bardow A., Jensen S., B., Nauntofte B. 2002. Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. Oral Dis. May; 8(3):117-29.

Penner, C., R, Muller S, 2006.Head and neck amyloidosis: a clinicopathologic study of 15 cases. Oral Oncol. Apr; 42(4):421-9.

Pentenero, M., Davico Bonino L.., Tomasini C., Conrotto D., Gandolfo S. 2006. Localized oral amyloidosis of the palate. Amyloid. Mar; 13(1):42-6.

Pepys, M.,B., Rademacher T.,W., Amatayakul-Chantler S., Williams P., Noble G.,E., Hutchinson W.,L., Hawkins P.,N., Nelson S.,R., Gallimore J.,R., Herbert J., et al 1994. Human serum amyloid P component is an invariant constituent of amyloid deposits and has a uniquely homogeneous glycostructure. Proc Natl Acad Sci U S A. Jun 7; 91(12):5602-6.

Pérez, P., Goicovich E., Alliende C., Aguilera S., Leyton C., Molina C., Pinto R., Romo R., Martinez B., González M., J. 2000. Differential expression of matrix metalloproteinases in labial salivary glands of patients with primary Sjögren's syndrome. Arthritis Rheum. Dec; 43(12):2807-17.

Petti, S., Scully C. 2009. Polyphenols, oral health and disease: A review J Dent. Jun; 37(6):413-23.

Philipp, A., Kralisch S., Bachmann A., Lossner U., Kratzsch J., Blüher M., Stumvoll M., Fasshauer M. 2010. Serum levels of the adipokine zinc- α2-glycoprotein are increased in chronic hemodialysis. Metabolism. 2011 May; 60(5):669-72.

Pijpe, J., Kalk W.,W., Bootsma H., Spijkervet F.,K., Kallenberg C.,G., Vissink A. 2007. Progression of salivary gland dysfunction in patients with Sjögren's syndrome. Ann Rheum Dis. Jan; 66(1):107-12.

Piludu, M., Lantini M., S., Cossu M., Piras M., Oppenheim F., G., Helmerhorst E.,J., Siqueira W., Hand A., R.2006.Salivary histatins in human deep posterior lingual glands (of von Ebner). Arch Oral Biol. Nov; 51(11):967-73.

Quintas, A., Vaz D., C., Cardoso I., Saraiva M., J., Brito R., M.2001.Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. J Biol Chem.Jul20; 276(29); 27207-13.

Ragunath, C., Manuel S., G., Venkataraman V., Sait H., B., Kasinathan C., Ramasubbu N.2008.Probing the role of aromatic residues at the secondary saccharide-binding sites of human salivary alpha-amylase in substrate hydrolysis and bacterial binding. J Mol Biol. Dec 31; 384(5):1232-48.

Rai, B., Kaur J., Catalina M., Anand S., C., Jacobs R., Teughels W. 2011. Effect of simulated microgravity on salivary and serum oxidants, antioxidants, and periodontal status. J Periodontol. Oct; 82(10):1478-82.

Redondo, C., Damas A., M., Olofsson A., Lundgren E., Saraiva M., J. 2000. Search for intermediate structures in transthyretin fibrillogenesis: soluble tetrameric Tyr78Phe TTR expresses a specific epitope present only in amyloid fibrils. J Mol Biol. Dec 1; 304/3):461.70.

Reed S., B., Morris G., T. 1992. Amyloidosis: current approaches for diagnosis and treatment. J Ky Med Assoc. Feb; 90(2):68-72.

Riad, M., Barton J.,R., Wilson J.,A., Freeman C.,P., Maran A.,G.1991.Parotid salivary secretory pattern in bulimia nervosa. Acta Otolaryngol.111(2):392-5.

Ribeiro do Rosário M., Antunes L., Barros F., 1961. Contribuição para o estudo clínico e laboratorial da paramiloidose de Corino de Andrade. J Soc Cien Méd. 75:1-46.

Robinovitch, M., R., Ashley R., L., Iversen J., M., Vigoren E., M., Oppenheim F., G., Lamkin M.2001. Parotid salivary basic proline-rich proteins inhibit HIV-1infectivity.Oral Dis. Mar; 7(2):86-93.

Rocha, N., Velho G., Horta M., Martins A., Massa A. 2005. Cutaneous manifestations of familial amyloidotic polyneuropathy. J Eur Acad Dermatol Venereol. Sep; 19(5):605-7.

Rukavina, J., G. Block W., D., Curtis, A., C.1956. Familial primary systemic amyloidosis: experimental, genetic, and clinical study. J Invest Dermatol. Sep; 27(3):111-31.

Russell, S., T., Tisdale M., J. 2005. The role of glucocorticoids in the induction of zinc-alpha2-glycoprotein expression in adipose tissue in cancer cachexia. Br J Cancer. Mar 14;92 (5): 876-81.

Said, G., Ropert A., Faux N. 1984. Length-dependent degeneration of fibers in Portuguese amyloid polyneuropathy: a clinicopathologic study. Neurology. Aug; 34(8):1025-32.

Salo, T., Lyons J., G., Rahemtulla F., Birkedal-Hansen H., Larjava H. 1991. Transforming growth factor-beta 1 up-regulates type IV collagenase expression in cultured human keratinocytes. J Biol Chem. Jun 25; 266(18):11436-41.

Sánchez, L., M., Chirino A., J, Bjorkman P. 1999. Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules. Science. Mar 19; 283(5409):1914-9.

Sánchez-Pérez, L., Golubov J., Irigoyen-Camacho M., E., Moctezuma P., A., Acosta-Gio E. 2009. Clinical, salivary, and bacterial markers for caries risk assessment in schoolchildren: a 4-year follow-up. Int J Paediatr Dent. May; 19(3):186-92.

Saporta, M., A., Zaros C., Cruz M., W., André C., Misrahib M., Bonaïti-Pellié C., Planté-Bordeneuvee V.2009. Penetrance estimation of TTR familial amyloid polyneuropathy (type I) in Brazilian families. Eur J Neurol. Mar; 16(3):337-41.

Saraiva, M.J, Costa P., P, Goodman D., S. 1983. Studies on plasma transthyretin (prealbumin) in familial amyloidotic polyneuropathy, Portuguese type. J Lab Clin Med. Oct; 102(4):590-603

Saraiva, M., J., Birken S., Costa P., P., Goodman D., S. 1984. Amyloid fibril protein in familial amyloidotic polyneuropathy portuguese type. Definition of molecular abnormality in transthyretin (prealbumin). J Clin Invest. July; 74(1):104-19.

Saraiva, M.,J., Costa P.,P., Almeida Mdo R., Banzhoff A., Altland K., Ferlini A., Rubboli G., Plasmati R., Tassinari C,.A,. Romeo G., et al. 1988. Familial amyloidotic polyneuropathy: transthyretin (prealbumin) variants in kindreds of Italian origin. Human Genet. Dec; 80(4):341-3.

Saraiva, M., J. Transthyretin mutations in hyperthyroxinemia and amyloid diseases.2001. Human Mutat. Jun; 17(6):493-503.Review.

Saraiva, M., M. Alterações da função ano-recto-cólica na polineuropatia amiloidótica familiar. 1999. Tese de Doutoramento. Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto.

Sasaky, H., Yoshioka N., Takagy Y., Sakaky Y. 1985. Structure of the chromosomal gene for human serum prealbumin. Gene.37 (1-3) 37:7.

Sawabe, M., Hamamatsu A., Ito T., Arai T., Ishikawa K., Chida K., Izumiyama N., Honma N., Takubo K., Nakazato M. 2003. Early pathogenesis of cardiac amyloid deposition in senile systemic amyloidosis: close relationship between amyloid deposits and the basement membranes of myocardial cells. Virchows Arch. Mar; 442(3):252-7.

Schenkels, L., C., Veerman E., C., I., Nieuw Amerong. A., V.1995. Biochemical composition of human saliva in relation to other mucosal fluids. Crit Rev Oral Biol Med.6 (2):161-75.

Schormann, N., Murrell J., R., Benson M., D.1998.Tertiary structures of amyloidogenic and non-amyloidogenic transthyretin variants: new model for amyloid fibril formation. Amyloid. Sep; 5(3):175-87.

Sekijima, M., Motono C., Yamasaki S,, Kaneko,K., Akiyama Y. 2003.Molecular dynamics simulation of dimeric and monomeric forms of human prion protein: insight into dynamics and properties. Biophys J. Aug; 85(2):1176-85.

Selikoff, I., J, Robitzek E., H. 1947. Gingival Biopsy for the Diagnosis of Generalized Amyloidosis. Am J Pathol. Nov; 23(6):1099-111.

Sequeiros, J., Saraiva M., J.1987.Onset in the seventh decade and lack of symptoms in heterozygotes for the TTRMet30 mutation in hereditary amyloid neuropathy-type I (Portuguese, Andrade). Am J Med Genet. Jun; 27(2):345-57.

Serpell, L., C., Sunde M., Blake C. C. 1997.The molecular basis of amyloidosis. Cell Mol Life Sci. Dec: 53(11-12):871-87.

Shiboski, C., H., Hodgson T., A. H.., Ship J., A., SchiØdt M. 2007.Management of salivary hypofunction during and after radiotherapy**.** Oral Surg Oral Med Oral Pathol Oral Radiol Endod. Mar; 103 Suppl: S66.e1-19.

Ship, J., A., Fischer D., J. 1997. The relationship between dehydration and parotid salivary gland function in young and older healthy adults. J Gerontol A Biol Sci Med Sci. Sep; 52(5):M310-9.

Shirasaki, S, Fujii H., Takahashi M., Sato T., Ebina M., Noto Y., Hirota K. 2007. Correlation between salivary alpha-amylase activity and pain scale in patients with chronic pain. Reg Anesth Pain Med. Mar-Apr; 32(2):120-3.

Siqueira, W., L., Oppenheim F., G.2009. Small molecular weight proteins/peptides present in the in vivo formed human acquired enamel pellicle. Arch Oral Biol. May; 54(5):437-44.

Sipe, J.,D., Benson M.,D., Buxbaum J.,N., Ikeda S., Merlini G., Saraiva M.,J., Westermark P. 2010. Amyloid fibril protein nomenclature: 2010 recommendations from the nomenclature committee of the International Society of Amyloidosis. Amyloid. Sep; 17(3-4):101-4.

Sissons, C., H., Cutress T., W. 1988. pH changes during simultaneous metabolism of urea and carbohydrate by human salivary bacteria in vitro. Arch Oral Biol. 33(8):579-87.

Skopouli, F.,N., Li L., Boumba D., Stefanaki S., Hanel K., Moutsopoulos H.,M., Krilis S.,A. 1998. Association of mast cells with fibrosis and fatty infiltration in the minor salivary glands of patients with Sjögren's syndrome. Clin Exp Rheumatol. Jan-Feb; 16(1):63-5.

Slavkin, H., C.1998. Toward molecularly based diagnostics for the oral cavity. J Am Dent Assoc. Aug; 129(8):1138-43.

Slomiany, B., L., Aono M., Murty V., L., N. Slomiany A., Levine M., J., Tabak L., A. 1982.Lipid composition of submandibular saliva from normal and cystic fibrosis individuals. J Dent Res. Oct; 6(10):1163-6.

Small, D., H., Williamson T., Reed G., Clarris H., Beyreuther K., Masters C., L., Nurcombe V. 1996. The role of heparan sulfate proteoglycans in the pathogenesis of Alzheimer's disease. Ann N Y Acad Sci. Jan 17; 777:316-321.

Smeland, S., Kolset S., O., Lyon M.., Norum K., R., Blomhoff R.1997.Binding of perlecan to transthyretin in vitro. Biochem J. Sep 15; 326(Pt 3):829-36.

Soares, M., L., Coelho T., Sousa A., Holmgren G., Saraiva M., J., Kastner D., L., Buxbaum J.,N.2004.Haplotypes and DNA sequence variation within and surrounding the transthyretin gene: genotype–phenotype correlations in familial amyloid polyneuropathy (V30M) in Portugal and Sweden. Eur J Hum Genet. Mar; 12(3): 225-37.

Soares, M., L., Coelho T., Sousa A., Batalov S., Conceição I., Sales-Luis M., L., Ritchie M., D., Williams S., M., Nievergelt C., M., Schork N., J., Saraiva M., J., Buxbaum J., N. 2005. Susceptibility and modifier genes in Portuguese transthyretin V30M amyloid polyneuropathy: complexity in a single-gene disease. Hum Mol Genet. Feb 15; 14(4):543-53.

Soares, M., S., Batista-Filho M., M., Pimentel M., J., Passos I., A., Chimenos-Kustner E. 2009. Determination of salivary glucose in healthy adults. Med Oral Patol Oral Cir Bucal. Oct 1; 14(10):e510-3.

Sobue, G., Nakao N., Murakami K., Yasuda T., Sahashi K., Mitsuma T., Sasaki H.,Sakaki Y., Takahashi A.1990.Type I familial amyloid polyneuropathy: a pathological study of the peripheral nervous system. Brain. Aug; 113(Pt 4):903-19.

Sousa, A., Andersson R., Drugge U., Holmgren G., Sandgren O., 1993. Familial amyloidotic polyneuropathy in Sweden: geographical distribution, age of onset, and prevalence. Hum Hered. Sep- Oct; 43(5):288-94.

Sousa, A., Coelho T., Barros J., Sequeiros J.1995.Genetic epidemiology of familial amyloidotic polyneuropathy (FAP) -type I in Póvoa do Varzim and Vila do Conde (north of Portugal). Am J Med Genet. Dec 18; 60(6):512-21.

Sousa, M., M., Yan S., D., Stern D., Saraiva M., J. 2000. Interaction of the receptor for advanced glycation end products (RAGE) with transthyretin triggers nuclear transcription factor kB (NFkB) activation. Lab Invest 80:1010-1110.

Sousa, M., M., Cardoso I., Fernandes R., Guimarães A., Saraiva M., J.2001a. Deposition of transthyretin in early stages of familial amyloidotic polyneuropathy: evidence for toxicity of nonfibrillar aggregates. Am J Pathol. Dec; 159(6):1993-2000.

Sousa, M., M., Du Yan S., Fernandes R., Guimarães A., Stern D., Saraiva M.,J. 2001b. Familial amyloid polyneuropathy: receptor for advanced glycation end products-dependent triggering of neuronal inflammatory and apoptotic pathways. J Neurosci. Oct 1; 21(19):7576-86.

Sousa, M., M., Ferrão J., Fernandes R., Guimarães A., Geraldes J., B., Perdigoto R., Tomé L., Mota O., Negrão L., Furtado A., L., Saraiva M., J. 2004.Deposition and passage of transthyretin through the blood-nerve barrier in recipients of familial amyloid polyneuropathy livers. Lab Invest. Jul; 84(7):865-73.

Sreebny, L., M., Zhu W., X.1996. The use of whole saliva in the differential diagnosis of Sjögren`s syndrome. Adv Dent Res. Apr; 10(1):17-24.Review.

Stamenkovic, I. 2003. Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol. Jul; 200(4):448-64.

Stenudd, C., Nordlund A., Ryberg M., Johansson I., Källestål C., Strömberg N. 2001. The association of bacterial adhesion with dental caries. J Dent Res. Nov; 80(11):2005-10.

Stokes, J., R., Davies G., A. 2007. Viscoelasticity of human whole saliva collected after acid and mechanical stimulation. Biorheology. 44(3):141-60.

Stoopler, E.,T., Sollecito T.,P., Chen S.,Y.2003.Amyloid deposition in the oral cavity: a retrospective study and review of the literature. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. Jun; 95(6):674-80.Review.

Streckfus, C., Bigler L., Navazesh M., Al-Hashimi I.2001.Cytokine concentrations in stimulated whole saliva among patients with primary Sjögren`s syndrome, secondary Sjögren`s syndrome, and patients with primary Sjögren`s syndrome receiving varying doses of interferon for symptomatic treatment of the condition: a preliminary study. Clin Oral Investig.Jun;5(2):133-5.

Suhr, O., Danielsson A., Holmgren G., Steen L.1994. Malnutrition and gastrointestinal dysfunction as prognostic factors for survival in familial amyloidotic polyneuropathy. J Intern Med. May; 235(5):479-85.

Tabak, L., A.1995.In defense of the oral cavity: structure, biosynthesis, and function of salivary mucins. Annu Rev Physiol.57:547-64.

Tada, T., Ohkubo I., Niwa M., Sasaki M., Tateyama H., Eimoto T.1991. Immunohistochemical localization of Zn-alpha 2-glycoprotein in normal human tissues. J Histochem Cytochem. Sep; 39(9):1221-6.

Takagaki, M., Honke K., Tsukamoto T., Higashiyama S., Taniguchi N., Makita A., Ohkubo I. 1994. Zn-alpha 2-glycoprotein is a novel adhesive protein. Biochem Biophys Res Commun. Jun 30; 201(3):1339-47.

Takahashi, L., Kimura Y., Y., i., Araky S., 1988. Pathology of familial amyloidotic polyneuropathy occurring in Kumamoto. In Isobe T., Araky A., Uchino F., Kito S., Tsubura.,(Eds). Amyloid and Amyloidosis. Plenum Press, New York, pp511-516

Talal, N. 1992. Sjögren's syndrome: historical overview and clinical spectrum of disease. Rheum Dis Clin North Am. Aug; 18(3):507-15.

Tashima, K., Ando Y., Tanaka Y., Uchino M., Ando.1995.Change in the age of onset in patients with familial amyloidotic polyneuropathy type I. Intern Med. Aug; 34(8):748-50.

Tavassoli, M., Brunel N., Maher R., Johnson N.,W., Soussi T.1998.p53 antibodies in the saliva of patients with squamous cell carcinoma of the oral cavity Int J Cancer. Oct 29; 78(3):390-1.

Tawara, S., Araki S., Toshimori K., Nakagawa H., Ohtaki, S.1981.Amyloid fibril protein in type I familial amyloidotic polyneuropathy in Japanese. J Lab Clin Med. Dec; 98(6):811-22.

Teixeira, P., F., Cerca F., Santos C., D., Saraiva M., J. 2006. Endoplasmic reticulum stress associated with extracellular aggregates: evidence from Transthyretin deposition in familial amyloid polyneuropathy. J Biol Chem. Aug 4; 281(31):21998-2003.

Tenovuo, J.1989. Nonimmunoglobulin defence factors in human saliva. In Human Saliva: Clinical Chemistry and Microbiology, Edited by J. Tenovuo. Boca Raton, FL: CRC Press. Vol. II, pp. 55–91.

Tenovuo, J. 1997. Salivary parameters of relevance for assessing caries activity in individuals and populations. Community Dent Oral Epidemiol; Feb; 25(1):82-6

Toyooka, K., Fujimura H., Ueno S., Yoshikawa H., Kaido M., Nishimura T., Yorifuji S.,YanagiharaT.1995.Familial amyloid polyneuropathy associated with transthyretin Gly42 mutation: a quantitative light and electron microscopic study of the peripheral nervous system. Acta Neuropathol. 90(5):516-25.

Trieger, N., Cohen N., Calkins A., S., Calkins E. 1960. Gingival biopsy as diagnostic aid in amyloid disease. Arch Oral Biol. Jan; 1:187-92.

Tsai, H., Bobek L., A.1998. Human salivary histatins promising anti-fungal therapeutic agents. Crit Rev Oral Biol Med. 9(4):480-97.Review.

Tucker, A., S.2007.Salivary gland development. Semin Cell Dev Biol. Apr; 18(2):237-44.

Tufvesson, E., Westergren-Thorsson G. 2000. Alteration of proteoglycan synthesis in human lung fibroblasts induced by interleukin-1beta and tumor necrosis factor-alpha. J Cell Biochem. Mar; 77(2):298-309.

Uemichi, T., Liepnieks J., J., Benson M., D.1994.Hereditary renal amyloidosis with a novel variant fibrinogen. J Clin Invest. Feb; 93(2):731-6.

Uemichi, T., Uitti R., J. Koeppen A., H., Donat J., R., Benson M., D. 1999. Oculoleptomeningeal amyloidosis associate with a new transthyretin variant Ser64.Arch Neurol. Sep; 56(9):1152-5.

van Stegeren, A., H., Wolf O., T., Kindt M. 2008.Salivary alpha amylase and cortisol responses to different stress tasks: Impact of sex. Int J Psychophysiol. Jul; 69(1):33–40.

van der Waal, I., Fehmers M.,C., Kraal E.,R. 1973. Amyloidosis: its significance in oral surgery. Review of the literature and report of a case. Oral Surg Oral Med Oral Pathol. Oct; 36(4):469-81.

van der Waal, N., Henzen-Logmans S., van der Kwast W.,A., van der Waal I. 1984. Amyloidosis of the tongue: a clinical and postmortem study Oral Pathol. Dec; 13(6):632-9.

Virchows, R.1853. Découverte dune substance qui donne lieu au memes reactions chimique que la cellulose vegetable dans le corps humain. C.R. Acad Sci. (Paris) 37: 492-493.

Vitorino, R., Lobo M., J., Duarte J., R., Ferrer-Correia A., J., Domingues P., M., Amado F., M.2005.The role of salivary peptides in dental caries. Biomed Chromatogr. Apr; 19(3):214-22.

von Bültzingslöwen, I., Sollecito T., Fox P., P., Daniels T., Jonsson R., Lockhart P.,B., Wray D., Brennan M.,T., Carrozzo M., Gandera B., Fujibayashi T., Navazesh M., Rhodus N.,L., Schiød M. 2007.Salivary dysfunction associated with systemic diseases: systematic review and clinical management recommendations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. Mar; 103Suppl:S57.e1.15.Review.

Waits, R., P., Yamada T., Uemichi T., Benson M., D.1995.Low plasma concentrations of retinol-binding protein in individuals with mutations affecting position 84 of the transthyretin molecule. Clin Chem. Sep; 41(9):1288-91.

Wall, J.,S., Richey .T, Stuckey A., Donnell R., Macy S., Martin E.,B., Williams A., Higuchi K., Kennel S.,J 2011. In vivo molecular imaging of peripheral amyloidosis using heparin-binding peptides. Proc Natl Acad Sci U S A. Aug 23; 108(34):E586-94.

Wallace, M.R., Conneally P.M., Benson M., D.1988.A DNA test for Indiana/Swiss hereditary amyloidosis (FAP II). Am J Hum Genet. Aug; 43(2):182-7.

Weber, H.1867.Mollities ossium, doubtful whether carcinomatous or syphilitic. Transactions of the Pathological Society of London, 18,206-210.

Wiklund, U., Hörnsten R., Karlsson M., Suhr O., B., Jensen S., M.2008. Abnormal heart rate variability and subtle atrial arrhythmia in patients with familial amyloidotic polyneuropathy. Ann Noninvasive Electrocardiol. Jul; 13(3):249-56.

Wilks, S. 1865.Report on lardaceous disease. Guy's Hospital Reports, 11, 45-55.

Wilson, C., Heppner K., Labosky P.,A., Hogan B.,L., Matrisian L.,M. 1997. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci U S A. Feb 18; 94(4):1402-7.

Wotman, S., Mercadante J., Mandel I.,D., Goldman R.,S., Denning C.1973.The occurrence of calculus in normal children, children with cystic fibrosis, and children with asthma. J Periodontol. May; 44(5):278-80.

Wu, Z., Van Ryk D., Davis C., Abrams W., R., Chaiken I., Magnani J., Malamud D. 2003. Salivary agglutinin inhibits HIV type 1 infectivity through interaction with viral glycoprotein 120. AIDS Res Hum Retroviruses. Mar; 19(3):201-9.

Yamamura, Y., Kito S., Shimoyama M., Katayana S., Nakano T., Anzai N., 1988. Pathology studies of familial amyloidosis. In: Isobe T., Araky A., Uchino F., Kito S., Tsubura E., (Eds). Amyloid and Amyloidosis. Plenum Press, New York, pp511-516

Yeaman, C., Grindstaff K., K., Nelson W., J. 1999. New perspectives on mechanisms involved in generating epithelial cell polarity. Physiol Rev. Jan; 79(1):73-98.Review.

Yoshioka, K., Furuya H., Sasaki H., Saraiva M.,J., Costa P.,P., Sakaki Y. 1989.Haplotype analysis of familial amyloidotic polyneuropathy. Evidence for multiple origins of the Val-Met mutation most common to the disease. Hum Genet. Apr; 82(1):9-13.

Zussman, E., Yarin A., L., Nagler R., M. 2007. Age- and flow-dependency of salivary Viscoelasticity. J Dent Res. Mar; 86(3):281-5.