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The significance of electron microscopic examination of gingiva in cases of Hunter syndrome and hereditary gingival fibromatosis

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Abstract

INTRODUCTION: Electron microscopy has been for decades a basic morphological method still used in diagnostic protocols of some pathological conditions affecting the ultrastructure of cells and extracellular matrix. The aim of this study was an ultrastructural description of gingiva of patients with Hunter syndrome and hereditary gingival fibromatosis. **PATIENTS AND METHODS:** Gingival biopsies were obtained during surgical periodontal treatment from a 9-year-old boy with Hunter disease (with enzyme replacement therapy with recombinant human idursulphase) and a 15-year-old girl with hereditary gingival fibromatosis. Gingival samples obtained from the upper anterior region were processed and examined with transmission electron microscope. **RESULTS**: In the case of Hunter syndrome due to the genetic lack of one lysosomal enzyme, an intercellular accumulation of glycosaminoglycans occurs. Within the gingiva of a patient with Hunter syndrome we observed membrane-bound storage vesicles in the cytoplasm of fibroblasts, endothelial cells of capillaries, surface epithelial cells, mast cells, and macrophages. Despite a long-term enzyme replacement therapy which improves clinical manifestations of Hunter syndrome, on the cellular level we still found marked accumulations of glycosaminoglycans in the cytoplasm of different cells as well as in the extracellular matrix. Hereditary gingival fibromatosis is a benign, slowly progressive and non-inflammatory gingival enlargement with a predominance of randomly oriented collagen fibrils in the gingival lamina propria. Some of these fibrils exhibited loops. Another unusual ultrastructural finding is the presence of empty perinuclear space in the cytoplasm of epithelial cells. The origin and significance of these non-membrane bound spaces are unknown. **CONCLUSION:** In both genetically determined diseases, the electron microscopic examination may be useful, and physicians get relevant information about the progress of illness.

ORIGINAL ARTICLE

INTRODUCTION

Hunter syndrome, also known as **mucopolysaccharidosis type II** (MPS2, OMIM 309900), is a rare X-linked recessive disorder that affects one in 140,000–156,000 live-born males in Europe (Scarpa *et al.* 2011). Hunter syndrome belongs to the group of inherited diseases called **lysosomal storage diseases**. It is caused by a mutation in the gene encoding lysosomal enzyme iduronate-2-sulphatase on chromosome Xq28. Physiologically, iduronate-2-sulphatase is responsible for the cleavage of O-linked sulphate moieties from the glycosaminoglycans dermatan sulphate and heparan sulphate as the first step in their degradation pathway. The deficiency of iduronate-2-sulphatase leads to the massive accumulation of glycosaminoglycans in many different tissues and organs, resulting in multi-system manifestations (Tylki-Szymańska *et al.* 2014). Recently, at least 530 mutations have been described that influenced clinical manifestations. Large alterations lead to a severe form of Hunter syndrome which is characterized by early onset, and first manifestations appear shortly after the age of 2 years. Missense mutations result in heterogeneous phenotypes ranging from the severe to the attenuated form, and they are characterized by late onset (Saito *et al.* 2016).

Hereditary gingival fibromatosis (HGF), also known as idiopathic gingival hyperplasia (GINGF1, OMIM 135300), is a rare hereditary condition that affects one in 750, 000 individuals and can occur in both genders and either of the jaws (Bittencourt *et al.* 2000). It is characterized by slowly progressive, non-haemorrhagic, fibrous enlargement of maxillary and mandibular keratinized gingiva caused by the increase in mucosal connective tissue elements, mostly associated with some syndrome (Dani *et al.* 2015). GINGF1 is caused by a heterozygous mutation in the SOS1 gene on chromosome 2p22. Other loci for gingival fibromatosis have also been found on chromosome 5q (Xiao *et al.* 2001), chromosome 2p23.3-p22.3 (Xe *et al.* 2005), and chromosome 11p15 (Zhu *et al.* 2007). More recently, an additional locus has been assigned to chromosome 2p16-p13 (Douzgou & Dallapiccola 2011). HGF may present in two forms: autosomal-dominant or less commonly autosomal-recessive mode of inheritance, as an isolated disorder or as part of a syndrome. Dominant forms are usually isolated (non-syndromic) and recessive ones are associated with a systemic disease or syndrome (Gawron *et al.* 2016).

Transmission electron microscopy (TEM), considered by some to be an old technique, was introduced by a German physicist Ernst Ruska and an electrical engineer Max Knoll as a unique laboratory technique in 1933. Yet, with the recent developments of light microscopy techniques that overcome the limitations imposed by the diffraction boundary, the question arises as to whether the importance of TEM is on the wane (Knott & Genoud 2013). Despite the mentioned fact of progress in optics as well as in new laboratory diagnostic methods, classic TEM (as a basic morphological tool) is still used in diagnostic protocols of some pathological conditions affecting the ultrastructure of cells and their extracellular matrix. TEM is routinely utilized during diagnosis of primary ciliary dyskinesia (Shoemark *et al.* 2012), damage to glomerular filtration barrier (Mandache & Penescu 2012), differential diagnosis of histologically similar neoplasms (Wick 2016) or rapid detection of some viruses (Goldsmith & Miller 2009; Hazelton & Gelderblom 2003).

The aim of this study was an **ultrastructural description of gingiva of patients** with Hunter syndrome and hereditary gingival fibromatosis. Both genetically determined diseases cause extreme **overgrowth of gingiva**. This may be not only aesthetic but also serious health problem making the mastication more difficult, or causing downgraded oral hygiene, halitosis ("bad breath") and resulting in the social isolation of patients.

PATIENTS AND METHODS

Patient 1 with Hunter syndrome. Our examined patient with Hunter syndrome is the second male descendant of healthy parents. His elder brother also suffered from Hunter syndrome of a severe form with significant clinical impairment and typical body appearance known as gargoylism. Although the early development of our patient after birth was uneventful, at the age of 2 years his behaviour impaired (distress, hyperactivity, aggression, interference with speech capability – articulation, etc.). Laboratory tests showed the deficiency of iduronate-2-sulphatase enzyme activity in white blood cells. The enzyme concentration was 0.035 nmol/mg over 24 hours, whereas its normal values range from 1.7 to 4.6 nmol/mg over 24 hours. When the boy was three years old, the enzyme replacement therapy with recombinant human iduronate-2-sulphatase (idursulphase) was initiated (Elaprase, Shire Human Genetic Therapies, Inc.). The dosage was 0.6 mg/kg intravenously administered once per week. This type of therapy improves clinical manifestations of Hunter syndrome. For example, even after a 3-month administration, the glycosaminoglycans concentration in urine roughly halved and after a 9-month treatment, the glycosaminoglycans concentration in urine was at about one-third value compared to the onset of treatment. A gingival sample from the patient was taken during a necessary stomato-surgical procedure at the age of 9 years after his parents had signed the informed consent.

Patient 2 with hereditary gingival fibromatosis. A patient at the age of 15 years was referred to the consultation at our Department of Stomatology with the marked overgrowth of gingiva. She was referred to our clinic by an orthodontist (dentofacial orthopaedist) as due to full-blown hyperplasia of gingiva with enormous bleeding her dentist could not indicate and perform a fixed multibracket therapy. Subjectively, the patient

complained of bleeding during consuming harder food and massive bleeding during home oral hygiene. Considering the patient's age, she also had personal psychological problems with her aesthetic appearance when smiling, and she suffered from heavy halitosis which she tried to compensate by daily oral hygiene resulting in further development of gingival bleeding. During the patient's treatment, we performed gingivectomy with a good result, and the patient was satisfied as well. After signing the informed consent by her parents, the samples of gingiva taken were processed for further electron microscopic examination.

The samples (gingival biopsies from the upper anterior region) for **electron microscopic examination** were fixed in a fresh solution of glutaraldehyde in phosphate buffered saline (pH 7.2) at the room temperature for 3 hours. Then, tissue samples with a fixative were rinsed with the cooled phosphate buffer with 10% saccharose, three times for 30 minutes and left sealed for one night in the refrigerator. For post-fixation, we used Millonig solution containing 1% osmium tetroxide (pH 7.2). After dehydration with ascended grades of ethanol, the tissue was transferred to an embedding medium through propylene oxide. The epoxy resin used in the experiment was Durcupan (Fluka, Switzerland). Polymerization took place at 60°C and lasted for three days. Subsequently, the specimen was cut with ultramicrotome. To obtain the highest contrast of the sections, uranyl acetate with Reynolds' solution was used. Such prepared samples were assessed with the FEI Morgagni 268D 100KV with tungsten filament (Czech Republic) transmission electron microscope, where resulting electron micrographs were recorded by a 4 MPxCCD digital camera.

RESULTS

Gingiva represents readily accessible biological material suitable for electron microscopic confirmation of glycosaminoglycans accumulation in the cytoplasm of various cellular populations. Figures 1–4 present the ultrastructure of gingiva of the examined patient **with Hunter syndrome** after six years of replacement therapy with idursulphase enzyme. In the case of Hunter syndrome due to the genetic lack of one lysosomal enzyme, intercellular accumulation of glycosaminoglycans occurs. We found abnormal storage vesicles in different types of cells within the gingiva. These membrane-bound vesicles (vacuoles or storage lysosomes) contain mostly electron-lucent content, less often with the granular or filamentous material (probably mucopolysaccharides). Storage vesicles were found in the cytoplasm of fibroblasts (*Fig. 1*), endothelial cells and pericytes of capillaries (*Fig. 2a*), mast cells (*Fig. 2b*), epithelial cells of the surface stratified squamous epithelium (*Fig. 2c*), and the most prominent accumulation of these vesicles was present in the cytoplasm of macrophages (*Fig. 2d*). Also, we observed ultrastructural changes in the structure of the extracellular matrix of lamina propria. The ground substance significantly dominates over the collagen fibres. Additionally, collagen fibres were poorly developed and did not form typical huge bundles. Oxytalan fibres typical for the periodontium were detectable, too (*Fig. 3*). The most interesting finding was the presence of numerous membrane-bound vesicles within the extracellular matrix. The diameter of these secretory vesicles was around 1 micrometre (*Fig. 4*) and probably they represent a secretory product of fibroblasts (*Fig. 1b and 1c*). The morphology of fibroblasts (*Fig. 1*) suggests their high synthetic activity, which is necessary for the production and maintenance of the extracellular matrix. They have a large and pale nucleus (predominance of euchromatin), and well-developed rough endoplasmic reticulum in their cytoplasm. The storage vesicles occupy about 5 to 30% of the cellular volume. Inside the vesicles, we found in some cases lipid droplets (bodies) and also myelin-like figures (so-called "zebra bodies"). In cases of surface lining epithelial cells and endothelial cells the presence of storage vesicles was less conspicuous, and these vesicles occupied less than 5% of their cellular volume. The most prominent morphological changes were observed in macrophages. The whole cytoplasm of these phagocytic cells was filled with vesicles and lipid bodies. Their mitochondria were enormously enlarged, and the intermembrane space between the inner and outer membrane of the nuclear envelope was markedly dilated (*Fig. 2d*). Please note that in most of the cases the exact types of the cells were difficult to identify since their cytoplasm was entirely filled with numerous vesicles. Additionally, in some other cells only part of the cytoplasm contained vesicles, while the others retained normal cytoplasmic organelles (*Fig. 1d*).

Hereditary gingival fibromatosis represents gingival enlargement resulting from a larger amount and accumulation of connective tissue. Fibrosis is characterised by the thinnish vascularised extracellular matrix, which is infiltrated by densely arranged collagen fibres. Collagen fibres formed the major part of the connective tissue of lamina propria, however, paradoxically they had a different arrangement as it is in clinically normal gingiva.

In fact, collagen fibres showed a "chaotic" arrangement, and they were not organised to thicker and parallelly running bundles. Particular collagen fibrils often created unusual loops and bends (*Fig. 5*). There were present proteosynthetically active fibroblasts among collagen fibres (*Fig. 5*), but also less active forms called fibrocytes. Surface epithelium was slightly thickened, whereas there were sporadically present migrating lymphocytes within epithelial cells (Fig. 6a). Among epithelial cells of the surface stratified squamous epithelium many strong intercellular connections (desmosomes) were found (*Fig. 6b*). A surprising finding was the cavity spaces inside the cytoplasm of epithelial cells present perinuclearly (*Figs. 7*). This empty cavity

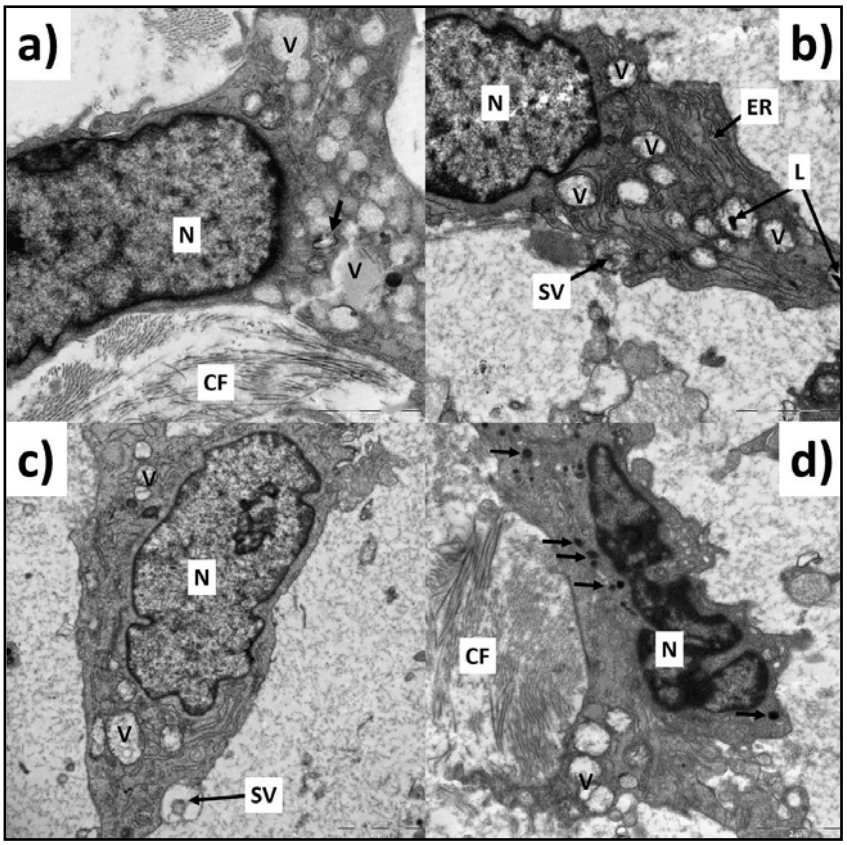

Fig. 1. Hunter syndrome – different fibroblasts from the connective tissue of gingival lamina propria. a) large and pale nucleus (N), the cytoplasm is filled with electron lucent storage vesicles (V) and with one lamellar body (arrow) resembling myelin figure. Around the cell a few thin collagen fibres (CF). Orig. Magn. 11,000x; b) large and pale nucleus (N), the cytoplasm is filled with well-developed rough endoplasmic reticulum (ER) and storage vesicles (V) occasionally containing lipid bodies (L). Some of these vesicles are actively secreted into the extracellular matrix as membrane-bound secretory vesicles (SV). Orig. Magn. 11,000x; c) nucleus with predominance of euchromatin and well visible nucleolus (N), within the cytoplasm some storage vesicles (V). Some of these vesicles are actively secreted into the extracellular matrix as membrane-bound secretory vesicles (SV). Orig. Magn. 8,900x; d) nonactive fibroblast with a nucleus (N) with predominance of heterochromatin, the cytoplasm is filled with electron lucent storage vesicles (V) and with lipid bodies (arrows). Around the cell a few thin collagen fibres (CF). Orig. Magn. 8,900x

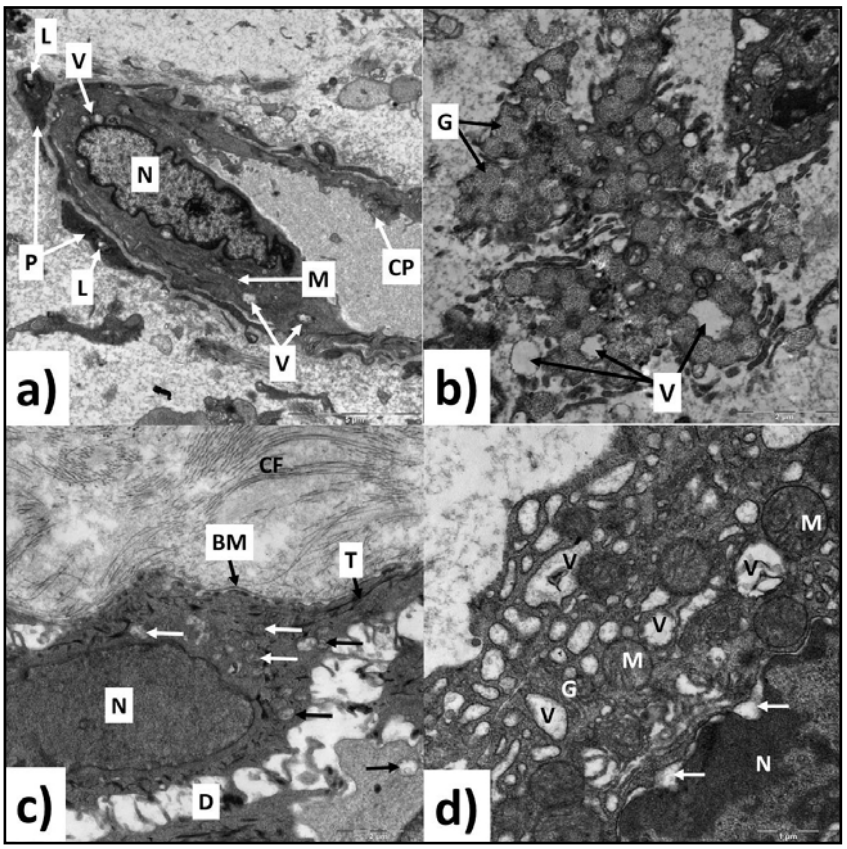

Fig. 2. Hunter syndrome - different cells of gingiva with storage vesicles inside cytoplasm. a) endothelial cell with the nucleus (N) contains in their cytoplasm elongated mitochondria (M) and a few storage vesicles (V). Toward to blood, their membrane creates typical cytoplasmic projections (CP). Partially, a pericyte is visible also (P) around the endothelial cell with lipid bodies-like storage vesicles (L). Orig. Magn. 5,600x; b) part of cytoplasm of mast cells, with numerous secretory granules (G) and some storage vesicles (V). Orig. Magn. 11,000x; c) basal epithelial cell from the surface epithelium with a large and pale nucleus (N) lies on the basement membrane (BM). Within the cytoplasm, storage vesicles (arrows) and intermediate filaments (tonofilaments, T) are visible. Between neighbouring epithelial cells there is a desmosome (D). Beneath the basement membrane, there are some collagen fibres of lamina propria located. Orig. Magn. 8,900x; d) part of the cytoplasm of macrophage. The nuclear envelope around the nucleus (N) has dilated the intermembranous space (arrows). The cytoplasm contains numerous Golgi apparatus (G), enlarged mitochondria (M) and an extremely large number of storage vesicles (V). Orig. Magn. 22,000x

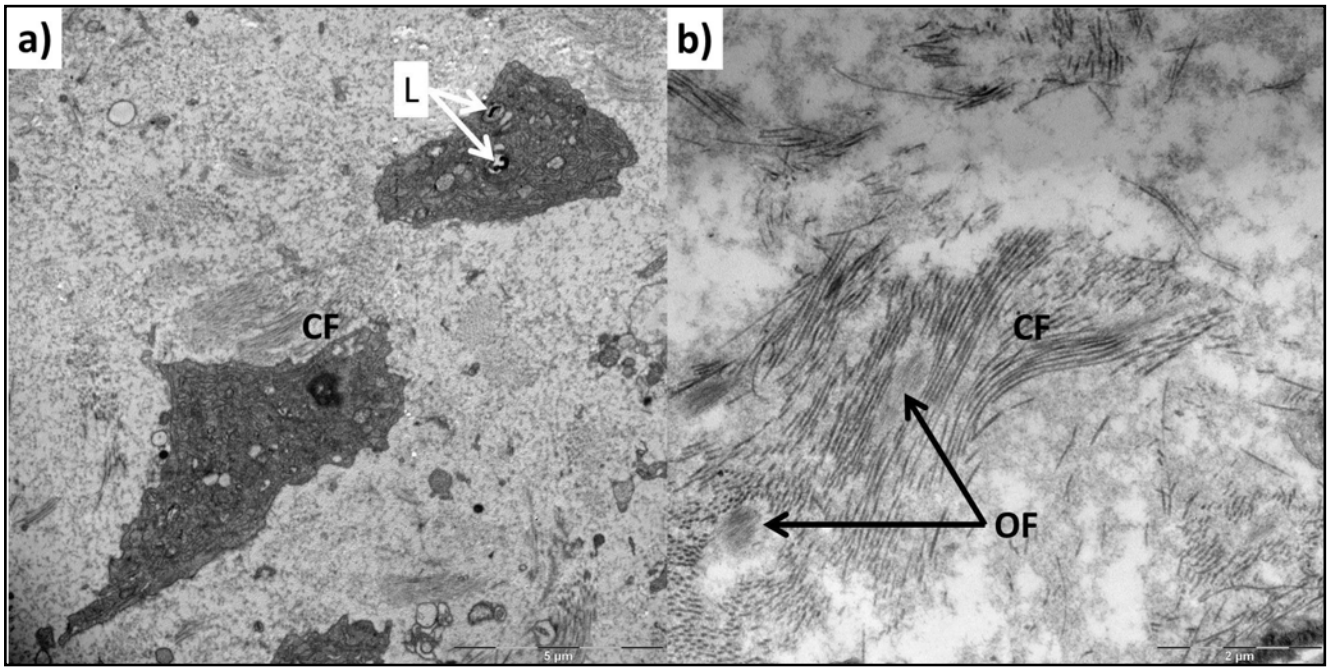

Fig. 3. Hunter syndrome – typically ultrastructure of the extracellular matrix of gingival lamina propria. a) two fibroblasts embedded in well-developed ground substance. Inside their cytoplasm "worm-like" lipid bodies (L). Collagen fibres are very poorly developed (CF). Orig. Magn. 5,600x; b) extracellular matrix with poorly developed collagen fibres (CF) and oxytalan fibres (OF). Orig. Magn. 11,000x

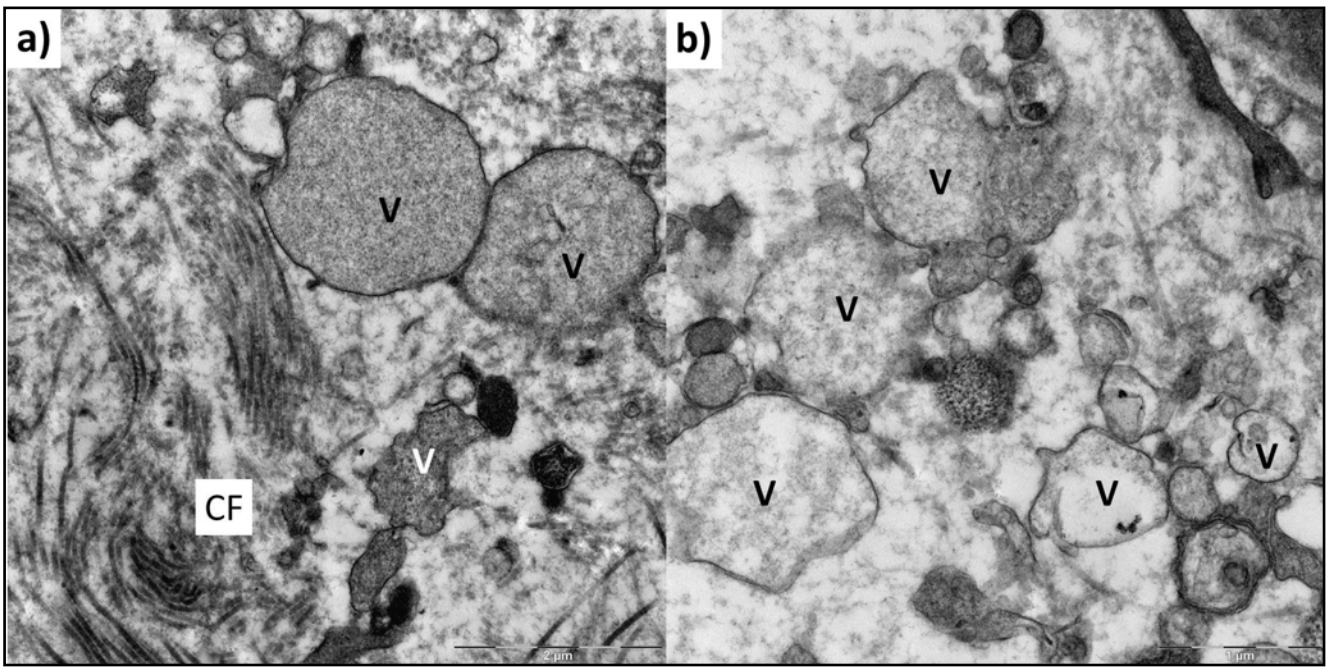

Fig. 4. Hunter syndrome – the extracellular matrix with large and heterogenous secretory vesicles (V) and poorly developed collagen fibres. a) Orig. Magn. 14,000x; b) Orig. Magn. 22,000x

space was not membrane-bound, and we are not able to explain either their origin or their role.

DISCUSSION

Examination of ultrastructures of tissues in patients with Hunter syndrome provides us with a picture of glycosaminoglycans accumulation in the cells and intercellular substance of various organs. Classic papers suggest metabolite accumulation in the form of vesicles particularly in the cytoplasm of cortical neurons or neurons of the myenteric plexus of the gut (Murphy *et al.* 1983). Intracellularly accumulated metabolites were visualized by electron microscopy in several ultrastructural studies performed on different organs, such as tonsils (Crow *et al.* 1983), kidney, pancreas, adrenal gland, testis, thyroid gland (Nagashima *et al.* 1976) or in hepatocytes and Kupffer cells of the liver (Yoshimoto

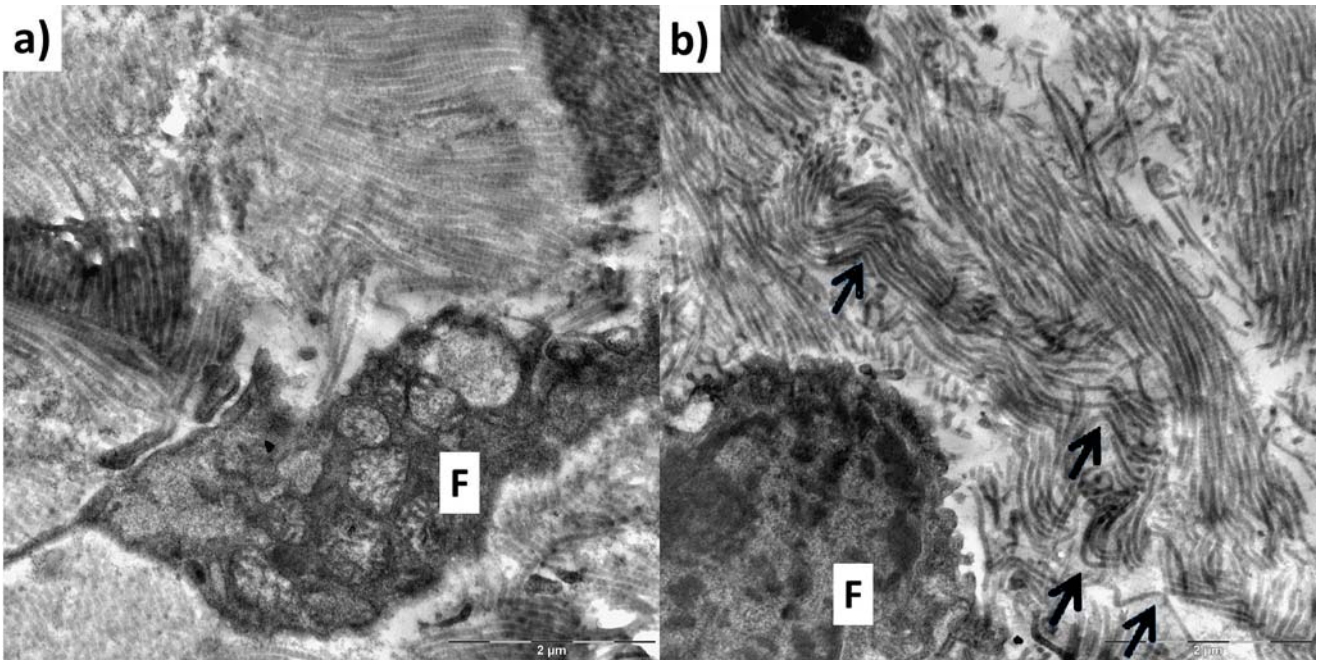

Fig. 5. Hereditary gingival fibromatosis – numerous collagen fibres around fibroblasts (F). Collagen fibrils create unusual loops and bends (arrows). a + b) Orig. Magn. 14,000x

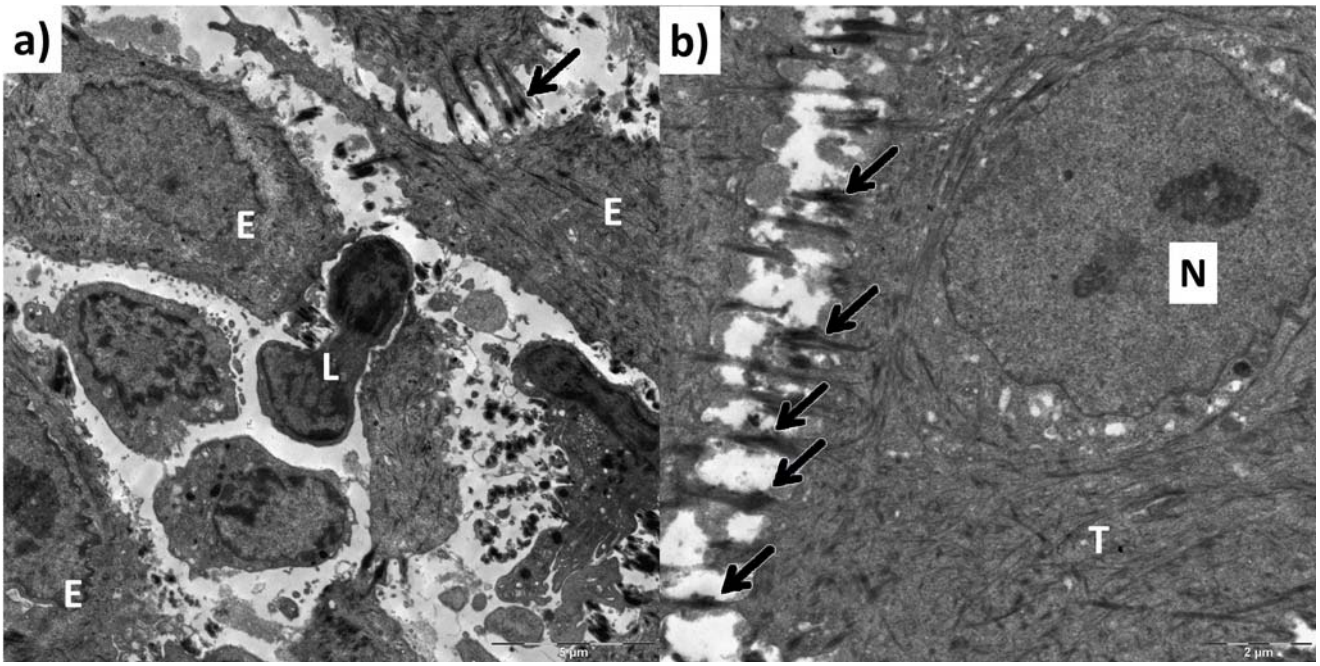

Fig. 6. Hereditary gingival fibromatosis – gingival surface stratified epithelium. a) among epithelial cells (E) is one lymphocyte (L). Orig. Magn. 4,400x; b) detail on epithelial cell with pale nucleus and prominent nucleolus (N), cytoplasm is filled by tonofilaments (T). Between epithelial cells numerous desmosomes (arrows). Orig. Magn. 7,100x

et al. 2006). Our results of electron microscopic examination of gingiva are very similar to the findings of Bioulac *et al.* (1975), who studied the ultrastructure of the skin of patients with Hunter syndrome. The similarity of both organs is evident since both investigated tissues are of the same embryonic origin and very similar histological structure. Through electron microscopy, it is possible to distinguish three types of vesicles, the presence of which we were able to observe as well. Type 1 represents a clear vesicle with the accumulation of glycosaminoglycans. Type 2 is called "zebra body" formed probably from ganglioside, and type 3 is an intermediate and mixed form of the type 1 and 2 (Oda *et al.* 1988).

The first ultrastructural studies demonstrating the correction of the metabolic defect after the exogenous addition of iduronate sulphate sulphatase in vitro (cul-

tured fibroblasts of Hunter patients) were published in the early 80s of the last century (Eliahu *et al.* 1981). The positive results of the enzyme substitution were morphologically visible in reducing the number of storage vesicles within the cytoplasm of cultured fibroblasts. On the other hand, unfortunately, due to newer papers, it is still evident that despite the latest enzyme replacement therapy the products of impaired metabolism still accumulate for example in aortic valves (Sato *et al.* 2013). Such ultrastructural changes in organs often cause lethal complications in patients with Hunter syndrome. We regret to say that also our morphologic research shows that in spite of substitutional enzyme therapy there still occurs prominent accumulation of metabolism products not only in different types of cells but also in the extracellular matrix.

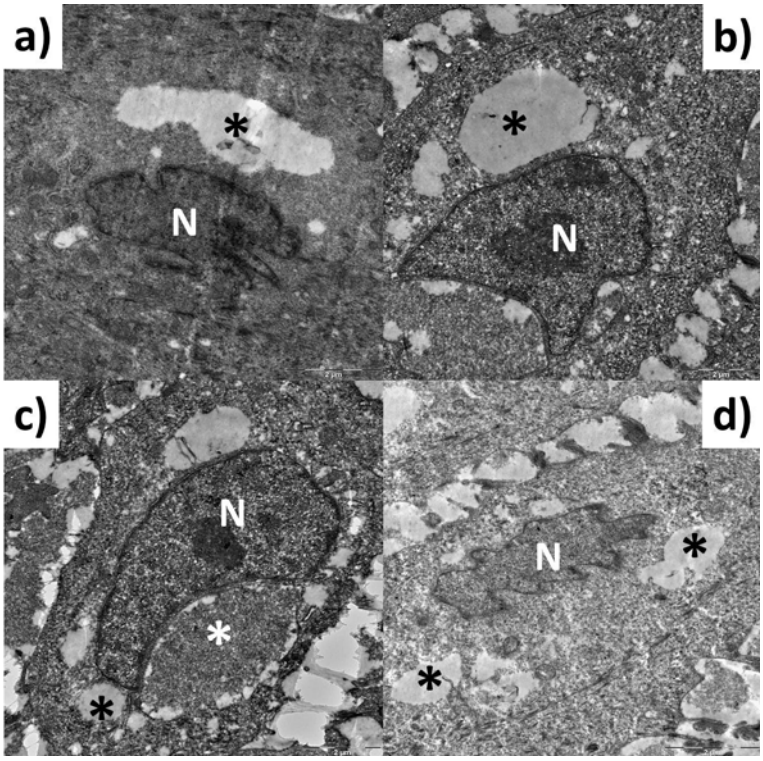

Fig. 7. Hereditary gingival fibromatosis – empty cavity spaces (asterisk) nearby nuclei (N) of epithelial cells. Orig. Magn. 8,900x

Hereditary gingival fibromatosis is a special type of benign, slowly progressive and non-inflammatory gingival enlargement (Livada & Shiloah 2012). There are some relatively different hypotheses about the pathogenesis of this gingival overgrowth. Kather *et al.* (2008) described that the collagen fraction was significantly greater in all HGF types compared with clinically healthy gingiva. Also, the number of fibroblasts was significantly increased in HGF types 1 and 2. Another histological study of HGF gingiva performed by Lee *et al.* (2006) confirmed increased numbers of fibroblasts (30%) and increased collagen fibres (10%). Additionally, the cited authors found increased fibroblasts proliferation under in vitro conditions, in both monolayer and three-dimensional matrix cultures. The observed increased gingival fibroblast proliferation is mediated by SOS1 gene mutation (Jang *et al.* 2007). Probably, not only fibroblasts but also myofibroblasts (expressed alpha smooth muscle actin) play an important role in the pathogenesis of HGF (Bitu *et al.* 2006). This hypothesis is not generally accepted, e.g. Sakamoto *et al.* (2002) immunohistochemically excluded the occurrence of myofibroblasts in the gingival connective tissue in the case of HGF. A different mechanism concerning the increased synthesis of collagen fibres was described by Noyan *et al.* (1994). According to the last-mentioned study, fibroblasts phagocytose the granules produced by mast cells; so mast cells are responsible for gingival hyperplasia. On the other hand, Meng *et al.* (2008) found that the interactions between surface keratinocytes and underlining fibroblasts contribute to the pathogenesis of HGF. It is surprising that the HGF is not typical only in humans. Similar hereditary hyperplasia of gingiva was also described in a wild European red fox (Schulze *et al.* 2008).

Most of ultrastructural studies concerning gingival samples of patients with HGF focused exclusively on the morphology of connective tissue elements. Our results about the ultrastructure of connective tissue in the case of HGF are similar with the results of Barros *et al.* (2001). A typical morphological sign of the connective tissue of HGF patients is the predominance of randomly oriented collagen fibrils. Some of these fibrils exhibited loops. But according to Pêgo *et al.* (2016), these loops from collagen fibrils are common findings also in the cases of normal gingiva. Fewer papers are published about the ultrastructure of the surface epithelium. An interesting finding is the presence of empty perinuclear space in the cytoplasm of epithelial cells. Similar spaces are visible in the electron micrograph published by Meng *et al.* (2008), but without any description. The role and origin of this non-membrane-bound space are at this moment an enigma.

CONCLUSION

In both genetically determined diseases, the electron microscopic examination may be useful, and physicians get relevant information about the progress of illness (especially in the case of Hunter syndrome). But on the other hand, these diseases cannot be determined by transmission electron microscopy alone. TEM is still an additional laboratory method of great significance and should be used together with other diagnostic methods. Using a TEM method in the patient with Hunter

syndrome we confirmed the presence of vesicles with glycosaminoglycans in the cytoplasm of various cellular populations despite a several-year enzyme substitutional therapy.

Conflict of interest. The authors declare that they have no conflicts of interest. The authors received no financial support for the research, authorship, and/or publication of this article.

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