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Contents

Original papers

Reviews

Evaluation of the cytotoxicity of selected conventional glass ionomer cements on human gingival fibroblasts

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Abstract

Background. Dentistry materials are the most frequently used substitutes of human tissues. Therefore, an assessment of dental filling materials should cover not only their chemical, physical, and mechanical characteristics, but also their cytotoxicity.

Objectives. To compare the cytotoxic effects of 13 conventional glass ionomer cements on human gingival fibroblasts.

Material and methods. The assessment was conducted using the MTT test. Six samples were prepared for each material. Culture plates with cells and inserts with the materials were incubated at 37°C, 5% CO_2, and 95% humidity for 24 h. Then the inserts were removed, 1 mL of MTT was added in the amount of 0.5 mg/1 mL of the medium, and the samples were incubated in the described conditions without light for 2 h. The optical density was measured with an absorption spectrophotometer at a wavelength of 560 nm.

Results. The cytotoxic effects of the Argion Molar was significantly stronger than the Fuji Triage ($p = 0.007$), Chemfil Molar ($p < 0.0001$), and Ionofil Molar AC Quick ($p < 0.001$). The Fuji IX GP and Fuji IX Extra had a significantly stronger adverse effect than the Chemfil Molar ($p = 0.014$, $p = 0.029$, respectively) and Ionofil Molar AC Quick ($p = 0.017$, $p = 0.034$, respectively). The cements from the low cytotoxicity group were significantly more toxic vs materials whose presence resulted in fibroblast growth ($p < 0.001$).

Conclusions. The research conducted indicates that, although the materials studied may belong to the same group, they are characterized by low, yet not uniform, cytotoxicity on human gingival fibroblasts. The toxic effects should not be assigned to a relevant group of materials, but each dentistry product should be evaluated individually.

Key words: dentistry, fibroblasts, glass ionomer cements

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Due to developments in science and technology, there are numerous products for the reconstruction of tooth tissues currently available on the dentistry market. According to Tilberg et al., dentistry materials are the most frequently used substitutes of human tissues.[1] They can affect surrounding structures either directly or indirectly when substances released by the fillings migrate through dentin channels into the pulp during the curing process and/or after it is completed. Therefore, the assessment of dental filling materials should cover not only their chemical, physical and mechanical characteristics, but also their biocompatibility, which is understood as a material's ability to function in live organisms and induce the appropriate tissue response.[2,3] Its measures include cytotoxicity, that is, the effect of a studied material on cell viability.[2] Cytotoxicity is a complex process, as there are numerous mechanisms causing functional and structural changes in cells and tissues.[2,4]

Glass ionomer cements were launched in the dentistry market in the 1970s.[5–8] Their composition was based on fluoroaluminosilicate glass and a liquid part, usually a water solution of polyalkenoic acid.[5–8] Unfortunately, because of their disadvantages, including low mechanical strength, long setting time and high sensitivity to moisture at the beginning of curing, the first products from this group were criticized by clinicians.[5–8] However, these materials had some indisputable advantages: chemical adhesion to mineralized tooth tissues, remineralization and antibacterial properties.[5–8] For these reasons, conventional glass ionomer cements (GIC) have been modernized. They are widely used in dentistry, particularly as materials for reconstructing missing hard tissues in deciduous teeth.

Literature data on the biocompatibility of glass ionomer cements is inconsistent.[9] Many authors suggest their high biocompatibility.[10–13] However, there are reports noting an adverse effect of these materials on live cells.[14–16] Differences in the results obtained by researchers may result from variability in research protocols.[17,18] For this reason, the aim of our study was to compare the cytotoxicity of currently available conventional glass ionomer cements in identical conditions and to verify contradictory reports on their biocompatibility.

Material and methods

Material sample preparation

The test was conducted for 13 conventional glass ionomer materials (GIC), including 2 reinforced with silver (MGIC) in color A3 (Table 1).

The cements studied were packed in capsules. They were prepared immediately before the test, in sterile conditions, using a crusher and shaker as specified by the manufacturer. The prepared materials were applied into plastic rings

Table 1. Materials tested in the study

Material	Manufacturer	Lot
Argion Molar	VOCO GmbH, Cuxhaven, Germany	0936172
Fuji IX GP	GC Corp., Tokyo, Japan	0904201
Fuji IX Extra	GC Corp., Tokyo, Japan	0904211
Riva Silver	SDI Ltd., Bayswater, Australia	G1005031
Fuji IX Fast	GC Corp., Tokyo, Japan	0812174
Fuji Triage	GC Corp., Tokyo Japan	0806051
Chemfil Molar	DENTSPLY De Trey GmbH, Kostanz, Germany	0817100
Ionofil Molar AC Quick	VOCO GmbH, Cuxhaven, Germany	0912304
Ketac Silver	3M ESPE AG, Seefeld, Germany	328538
Ketac Molar Aplicap	3M ESPE AG, Seefeld, Germany	366335
Riva SC	SDI Ltd., Bayswater, Australia	A1004292
Ketac Molar Quick	3M ESPE AG, Seefeld, Germany	387995
Ketac Fil Plus Aplicap	3M ESPE AG, Seefeld, Germany	365422

of 5 mm (inner diameter) × 5 mm (height). The rings with the materials were placed in inserts (Nunc GmbH&Co KG, Wiesbaden, Germany), of a surface area of 0.47 cm^2 and pore diameter of 0.4 µm, which were located in 24-well culture plates (Nunc GmbH&Co KG, Wiesbaden, Germany) containing human gingival fibroblasts. Six samples were prepared for each material. Six wells with inserts without any material constituted the control.

Cell culture preparation

Human gingival fibroblasts from the adherent permanent cell line (ATCC® CRL-2014HGF-1 (LGC Promochem, Warszawa, Poland) were grown in Falcon containers (growth area of 75 cm^2) on DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, Warszawa, Poland) with 10% fetal bovine serum (FBS) (Gibco, Warszawa, Poland) added, at 37°C, 5% CO_2, and 95% humidity. When the confluent growth was obtained, the cells were incubated with 0.25% trypsin solution with 0.53 mM EDTA added. Then, a medium with 10% FBS was added to inhibit enzyme activity. The cell suspension diluted in a fresh medium was inoculated in 24-well plates and incubated for 24 h.

Cytotoxicity evaluation

The cytotoxicity of the materials studied was evaluated using the MTT test. It is an indirect method determining cell viability and proliferation on the basis of mitochondrial succinate dehydrogenase activity. In live cells, this enzyme reduces yellow tetrazole salt,

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, to formazan precipitating as insoluble grey-purple crystals. The intensity of the solution color after dissolving the crystals, measured by a spectrophotometer, is a measure of cell viability. For low cell survival, low enzymatic activity is found resulting in a low content of purple formazan and lower optical density values.

The culture plates with the cells and applied materials were incubated at 37°C, 5% CO_2, and 95% humidity for 24 h. Then, the inserts with the materials were removed, and 1 mL of medium containing 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to each well at a level of 0.5 mg/mL, and the plates were incubated without light in the conditions described above for 3 h. Afterwards, the fluid was aspirated from the culture, and 1 mL of isopropanol acidified with hydrochloric acid was added. To dissolve the formazan crystals, the solution obtained was stirred for a short time. The optical density (OD) was measured with a double-beam absorption spectrophotometer Lambda EZ 2001 (Perkin Elmer, Waltham, USA) at a wavelength of 560 nm. Cell viability was calculated using the following formula: [mean OD of test group/mean OD of control group] × 100%.

Cell viability was scored according to the method by da Silva et al.[19] If cell viability exceeded 90%, the material was deemed non-cytotoxic. For cell viability at the 60–90% range, the material was regarded as slightly cytotoxic. For cell viability at the 30–59% range, the material was regarded as moderately cytotoxic. For cell viability below 30%, the material was considered severely cytotoxic.

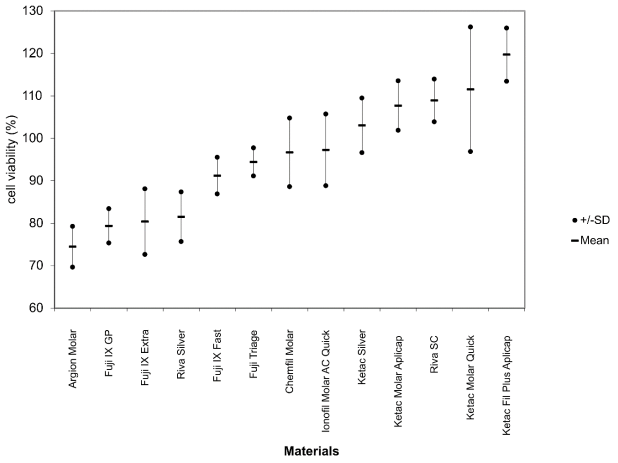
Statistical analysis

The statistical analysis was performed using the STATISTICA v. 8.0 (StatSoft Sp. z o.o., Kraków, Poland) software package. For comparisons between the groups, the one-way analysis of variance ANOVA and post hoc Tukey's HSD (honestly significant difference) tests were used. The hierarchical cluster analysis with a dendrogram, using the average linkage between groups, was used as the classification method. The level of significance was set at $p < 0.05$.

Results

None of the 13 materials exhibited high or medium cytotoxicity. In all cases, fibroblast survival exceeded 60%. The group with low cytotoxicity consisted of 4 glass ionomer cements: conventional – Fuji IX GP and Fuji IX Extra; and silver-reinforced – Argion Molar and Riva Silver. Other materials studied had no adverse effects. More than 90% of fibroblasts survived in the presence of Fuji IX Fast, Fuji Triage, Chemfil Molar, and Ionofil Molar AC Quick; while in the vicinity of Ketac Silver, Ketac Molar Aplicap, Riva SC, Ketac Molar Quick, and Ketac Fil Plus Aplicap, they multiplied.

Fig. 1. Mean cell viability ratios and standard deviations of the materials tested



One-way variance analysis indicated significant differences in cell survival with different materials ($p < 0.001$). Assessment with Tukey's post hoc test showed that the effect of materials with low cytotoxicity did not differ significantly. A similar relationship applied to the group of materials without an adverse effect on fibroblasts.

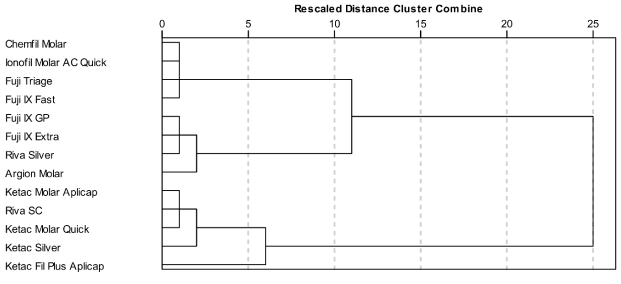
Significant differences were found between materials from different groups (Fig. 1).

The cytotoxic effect of Argion Molar was significantly stronger than Fuji Triage ($p = 0.007$), Chemfil Molar ($p < 0.0001$) and Ionofil Molar AC Quick ($p < 0.001$). Fuji IX GP and Fuji IX Extra had a significantly stronger adverse effect than Chemfil Molar ($p = 0.014$ and $p = 0.029$, respectively) and Ionofil Molar AC Quick ($p = 0.017$ and $p = 0.034$, respectively). Cements from the low cytotoxicity group were significantly more toxic vs materials whose presence resulted in fibroblast growth ($p < 0.001$).

The dendrogram (Fig. 2) presents three separate clusters of materials that are most similar to each other in terms of cell survival ratio in the culture.

The tested materials with the most similar cytotoxicity are connected by vertical lines and form a cluster. The position of the lines on the scale (at the top of the figure) indicates the distances between clusters: the closer to the scale center, the greater similarity in cytotoxicity.

Fig. 2. Similarities in the toxic effect of the materials examined



We found the greatest similarity in effects on the viability of fibroblasts in the following groups: Chemfil Molar (96.7%), Ionofil Molar AC Quick (97.2%), Fuji Triage (94.4%) and Fuji IX Fast (91.2%) in the first cluster; the second cluster – Fuji IX GP (79.4%), Fuji IX Extra (80.4%), Riva Silver (81.5%); and the third cluster consisted of Ketac Molar Aplicap (107.7%), Riva SC (108.9%), Ketac Molar Quick (111.5%), and Ketac Silver (103.0%).

Discussion

Studies evaluating the biological characteristics of dentistry materials have been conducted for many years. They have used various methods, including in vitro tests on cell cultures, pre-clinical studies on laboratory animals and clinical trials in patients.[2–4] The use of animals for biocompatibility assessments of dentistry materials represents an ethical problem and has been widely debated. For this reason, the International Organization for Standardization (ISO) recommends in vitro studies with cell cultures and limiting tests conducted on animal models.

Cytotoxic activity can be determined using various laboratory tests. The MTT test is recommended by ISO as a reference, and was used in our experiment. It determines the activity of succinate dehydrogenase, a mitochondrial enzyme present in live cells.[19]

Selection of an appropriate cell line is a very important part of the study during in vitro cytotoxicity assessments. Researchers' opinions on that issue vary. The use of permanent, standard cell lines or primary cells collected from gingiva, periodontium or the pulp is recommended. Permanent cell lines are morphologically and physiologically uniform. Primary cells represent clinical conditions better, but are diversified and have lower viability.[20] TheISO 10993 standard, standardizing in vitro studies, supports the use of permanent cell lines.[2,4,12] In our study, we used a standard human gingiva fibroblast line; and to reproduce conditions similar to clinical, we placed samples of materials on a semi-permeable membrane of inserts.

The study conducted indicates that the evaluated materials were characterized by low or lack of cytotoxicity. Schedle et al. obtained completely different results.[16] Using a flow cytometry method, the authors compared the cytotoxicity of various dentistry materials, including 2 conventional glass ionomer cements. On the basis of the study results, they suggested that for the evaluated cements, the adverse effects on fibroblasts were comparable to the effect of composite materials considered to be very cytotoxic. An unfavorable opinion on the biological characteristics of glass ionomer cements was presented in the study by Milhem et al.[14] They conducted an experiment using Artemia Salina larvae exposed to alcohol eluates of the assessed materials. The results indicated that Ketac Fil cement was more toxic than the composite materials.

In a majority of the published studies, the authors evaluating the biological effects of conventional glass ionomer cements showed that they were characterized by low cytotoxicity.[10–13] The results of our study are consistent with that finding. The group with low toxicity contained 4 materials: Argion Molar and Riva Silver (conventional glass ionomer cements reinforced with silver), and Fuji IX GP and Fuji IX Extra, differing significantly from the remaining 9 products.

The reasons for the cytotoxicity of the evaluated preparations have not been sufficiently explained. The literature contains various interpretations of this phenomenon. The authors emphasize the effect of low pH during setting and the effects of various released components.[17,21–23] Migration of certain ions is most often mentioned. The release of fluoride ions from glass-ionomer cements is commonly known.

Kan et al. investigated the cytotoxicity and fluoride release of 2 resin-modified GICs, a conventional glass-ionomer cement, and a resin composite.[24] Fluoride release and cytotoxicity were correlated, although the fluoride release did not account for the cytotoxicity observed. In their opinion, there were factors other than fluoride responsible for the cytotoxicity. According to Wilson et al., the following ions are released from conventional glass-ionomer cements: F, Na and Si.[25] Recent studies by Nicholson et al. have also demonstrated the release of Al, P, and Ca, depending on the pH solution.[26] The effect of different ions on the cytotoxicity of these materials is still unclear. In the authors' opinion, ions, apart from aluminum, are acceptable in the body and useful for a variety of physiological processes.[26] However, the total amount of aluminum released from glass-ionomer cements is so low that this is not a significant problem.[27] As indicated by Forss,[28] the ion amount leached to the environment is associated with the material's composition. In Tyas' opinion, contemporary glass-ionomer cements consist of fluoroaluminosilicate glass, usually a strontium or calcium salt, and polyalkenoic acid liquid, for example polyacrylic, maleic, itaconic, and tricarballylic acids.[6] The exact chemical compositions of the materials are not provided by the manufacturers.

Stanisławski et al. studied the cytotoxic effects of several ions released from different materials.[23] That study indicates that the F^-, Al^{3+} and Sr^{2+} levels were too low to damage cultured cells. In these authors' opinion, the main factors responsible for cytotoxicity were the presence of copper and silver ions in the case of HiDense – a conventional glass ionomer cement reinforced with silver. In our experiment, we did not study ion release, but the cytotoxicity of Argion Molar and Riva Silver may be related to a similar mechanism.

The studies by Stanisławski et al. and Soheili Majd et al. expanded our knowledge on biochemical mechanisms underlying the cytotoxicity of glass ionomer cements.[15,29] The above-mentioned authors proved that Fuji II and

Ketac Fil Plus Aplicap caused a reduction in cellular glutathione (GSH), one of the most important antioxidants in living organisms. The process of cellular GSH reduction is not fully known yet. Oxidative stress can be a possible cause underlying that phenomenon, possibly dependent on the presence of even small quantities of aluminum and/or iron ions.[23,29]

The results of laboratory experiments should not be directly extrapolated to clinical conditions. According to many authors, during tooth tissue reconstruction, the thickness and permeability of dentine remaining in the cavity should also be considered.[30] Forming a partial barrier, it can reduce the cytotoxic potential of the materials by limiting the availability of water required for hydrolysis of the released components (reduced diffusion) and buffering capacity of hydroxyapatites.

Our research indicates that although the studied materials may belong to the same group, they are characterized by low, yet not uniform, cytotoxicity. This is probably related to differences in their chemical composition, which remains a trade secret of the manufacturers.

Conclusions

It seems fair to conclude that a toxic effect should not be assigned to a relevant group of materials, but each dentistry product should be evaluated individually.

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The angiotensin II receptors type 1 blockage affects the urinary bladder activity in hyperosmolar-induced detrusor overactivity in rats: Preliminary results

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Abstract

Background. Angiotensin II receptors play a role in the pathogenesis of urinary bladder dysfunction, especially in the case of bladder outlet obstruction. The function of these receptors in the detrusor overactivity (DO) still remains unclear.

Objectives. The study aims to investigate some of the mechanisms through which hyperosmolarity induces urinary bladder overactivity. The effect of angiotensin II receptor type 1 – AT1 (telmisartan) on urinary bladder function in physiological state and in hyperosmolar-induced DO in rat model was explored.

Material and methods. Experiments were performed on 32 female Wistar rats. DO was induced by hyperosmolar saline intravesical instillation. Surgical procedures and cystometry were performed under urethane anesthesia. The measurements represent the average of 5 bladder micturition cycles. We analyzed: basal pressure, threshold pressure, micturition voiding pressure, intercontraction interval, compliance, functional bladder capacity, motility index and detrusor overactivity index.

Results. Intravesical hyperosmolar saline instillation induced DO. Telmisartan diminished the severity of hyperosmolar-induced DO. We observed a statistically significant increase of intercontraction interval (55%), functional bladder capacity (54%), compliance (140%). Also, a statistically significant decrease of detrusor overactivity index (18%) and motility index (9%) were observed. The difference of basal pressure, threshold pressure and micturition voiding pressure were not statistically significant. Moreover, telmisartan has no effect on urodynamic parameters in naïve rats.

Conclusions. Detrusor overactivity due to intravesical increased osmolarity seems to be at least partially mediated by AT1 receptors activity. On one hand, telmisartan diminished the severity of hyperosmolar-induced DO, and, on the other hand, has no effect on urodynamic parameters in naïve rats.

Key words: rat, overactive bladder, telmisartan, osmolarity, cystometry

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Urinary bladder function is regulated by the somatic and autonomic nervous system (ANS). With the exception of the cholinergic (via muscarinic receptors) and the adrenergic branch of ANS (via α and β receptors), the non-cholinergic/non-adrenergic mechanisms (NCNA) play an important role in proper urine storage and voiding. A wide range of neurotransmitters of the NCNA branch of ANS (e.g. SP – substance P, CGRP – calcitonin gene related peptide, ATP – adenosine 5'-triphosphate, VIP – vasoactive intestinal polypeptide, NY – neuropeptide Y, somatostatin, bombesin, etc.) act as stimulatory or inhibitory neuromodulators of adrenergic, cholinergic as well as purinergic transmission of lower urinary tract.[1] NCNA mechanisms implicated in the urethral sphincter action have been described previously. Phull et al.[2] observed that the blockade of angiotensin II receptors type 1 (AT1) and type 2 (AT2) decreases urethral resistance in stress urinary incontinence rat model. Moreover, angiotensin II treatment improves urethral tone in sphincter deficiency in the rat model. On the other hand, Tanabe et al.[3] showed that the AT1 receptors, rather than AT2 receptors, mediate angiotensin – induced smooth muscle rat urinary bladder strips contractions in in vitro studies. In the literature there is still sparse data regarding the role of the renin-angiotensin-aldosteron system in lower urinary tract function. Animal studies showed that estrogen deprivation leads to voiding dysfunction and urethral hypermobility. The main pathomechanisms for this seem to be increased angiotensin-converting enzyme (ACE) activity and up-regulation of AT1 and AT2 receptors.[4] Furthermore, Cho et al.[5] described the role of AT1 receptors in the development of detrusor overactivity (DO) due to bladder outlet obstruction (BOO) in a rat model. The function of AT1 receptors in DO remain unclear.

Therefore, we examined the effect of angiotensin II receptor type 1 – AT1 (Telmisartan) on urinary bladder function in the physiological state and in hyperosmolar-induced detrusor overactivity in a rat model.

Material and methods

Animals

Experiments were performed on 32 adult female Wistar rats (weight: 200–250 g). Rats were housed individually in cages. The animal room was maintained at a constant temperature (23°C), humidity and a 12:12 h alternating light-dark cycle. They were fed with animal food (Labofeed; Kcynia, Poland) without any restriction to water. The study has been approved by the Local Animals Ethical Committee.

Experimental groups

Thirty two animals were divided randomly into 5 groups: I (control) – healthy rats (n = 12), II – rats with hyperosmolar induced DO (n = 6), III – rats with hyperosmolar induced DO and intravesical administration of telmisartan (n = 6), IV – healthy rats with intravesical administration of telmisartan (n = 6), V – healthy rats with intravesical administration of DMSO solution used for telmisartan preparation (n = 2).

Anesthesia

All the surgical procedures and urodynamic studies were performed under anesthesia using intraperitoneal injection of 1.2 g/kg urethane (Sigma-Aldrich, St. Louis, USA).[6]

Detrusor overactivity (DO) induced by hyperosmolar intravesical stimulation

The neural reflex transmitted by unmyelinated afferent C fibers is crucial in DO development. Hyperosmolar stimuli activate these nerves via vanilloid receptors leading to increased local effector activity of C fibers. High concentrated urine penetrates the submucosal layers of the urinary bladder and activates capsaicin-sensitive C neurons and consequently, inducing neurogenic inflammation, which leads to DO.[7] The water deprivation for 16 h is sufficient to determine urine concentrating ability of kidneys. The urine concentration tests in female rats revealed that mean urine osmolarity was 2080 mOsm/L.[8] Hypertonic saline within physiological osmolarity range induces concentrated-dependent DO. Therefore, DO was induced by a continuously intravesical infusion of hypertonic saline solution (in physiological range at 2080 mOsm/L) at a rate of 0.046 mL/min.[9]

Drugs

Telmisartan (Sigma-Aldrich, Germany), a non-peptide AT1 angiotensin II receptor antagonist, was used in the following study. Telmisartan was dissolved in DMSO and in 0.9% saline to a final concentration of 3 mg/kg per dose. Under urethane anesthesia, the bladder was catheterized through the urethra and emptied. The telmisartan solution at 0.3 mL final volume was gently injected through the catheter (group III and IV) at a rate of 0.15 mL/min, and subsequently was left in contact with the urinary bladder mucosa for 15 min. Then the bladder was emptied again and flushed out using 0.5 mL 0.9% saline at a rate of 0.15 mL/min.[5,10]

Surgical procedure

Bladder catheter implantation: under urethane anesthesia, the abdomen was opened through a midline incision, and the bladder end of the polyethylene catheter (o.d. 0.97 mm/i.d. 0.58 mm; BALT, Poland) was passed through a 1 mm incision at the apex of the bladder dome and secured in place by a silk ligature 4-0, as previously described.[10]

Urodynamic studies

Cystometry was performed under urethane anesthesia after a 1 h recovery period from the surgical procedure. A room temperature solution was infused at a rate of 0.046 mL/min continuously into the urinary bladder. The free end of the implanted catheter was connected via a T-stopcock to a pressure transducer (UFI, Morro-Bay, USA) and injection pump (Unipan340A, Poland). Cystometry was recorded using PowerLab/8SP (ADInstruments, Castle Hill, Australia) set, as previously described.[10,11]

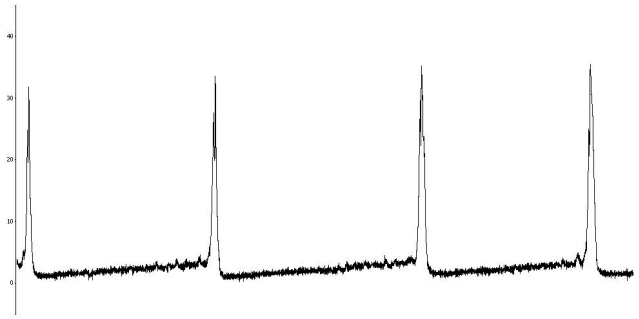
Experimental protocol

All animals underwent cystometry using isoosmolar (308 mOsm/L) saline solution (group I); hyperosmolar saline solution (group II); hyperosmolar saline after telmisartan solution administration (group III); isoosmolar saline after telmisartan solution administration (group IV); and isoosmolar saline solution after DMSO solution administration. DMSO solution was composed of 0.9% saline (75%), DMSO (25%) (the volume participation of individual components in solvent expressed in the per cent, is provided in square brackets). The measurements, which were repeated in each animal, represent the average of 5 bladder micturition cycles, after obtaining repetitive voiding. The following cystometrogram parameters were recorded: BP – basal pressure (cm H_2O), PT – threshold pressure (cm H_2O), MVP – micturition voiding pressure (cm H_2O), ICI – intercontraction interval [min.], compliance (mL/cm H_2O), fBC – functional bladder capacity [mL], MI – motility index (cm H_2O × s/min) in 10-minute intervals, DI – detrusor index (cm H_2O/mL) (in group I and IV) and DOI – detrusor overactivity index (cm H_2O/mL) (in group II and III), depicted as a quotient of the sum of amplitudes of all detrusor contractions during the filling phase and functional bladder capacity.[11] The intravesical administration of DMSO solution has no significant effect on motor and sensory urinary bladder activity (group V), thus this group was excluded from further analysis.

Statistical analysis

The results are expressed as mean and standard deviation (±SD). The Kruskal-Wallis test was used to compare the groups and a post hoc multiple comparison test was used for statistically significant results. Statistical significance was set at $p \leq 0.05$ for all tests.

Fig. 1. Cystometry in control rats



The figure shows 20-min interval – horizontal axis. Vertical axis estimates intravesical pressure of (-5) – 45 cm H_2O range.

Table 1. Cystometric parameters of control rats (group I), hyperosmolar-induced detrusor overactivity (group II), hyperosmolar-induced detrusor overactivity after intravesical telmisartan administration (group III), control after intravesical telmisartan administration (group IV)

Cystometric parameters	Group I control	Group II hyperosmolar-induced detrusor overactivity	Group III hyperosmolar-induced detrusor overactivity + Telmisartan	Group IV control + telmisartan	p-value
Basal pressure (BP) [cm H_2O]	1.41 ±0.60	3.07 ±0.16*	3.20 ±0.19	2.45 ±0.79	*0.01 vs group I
Threshold pressure (PT) [cm H_2O]	5.70 ±1.22	6.12 ±0.26	5.12 ±0.68	5.40 ±1.08	ns
Micturition voiding pressure (MVP) [cm H_2O]	27.40 ±4.90	25.90 ±4.90	29.15 ±3.62	30.12 ±2.38	ns
Intercontraction interval (ICI) [min]	5.28 ±1.55	2.59 ±0.26*	4.02 ±0.47**	5.70 ±2.13	*0.001 vs group I **0.002 vs group II
Compliance [mL/cm H_2O]	0.059 ±0.019	0.040 ±0.008	0.096 ±0.011**	0.045 ±0.012	**0.005 vs group II
Functional bladder capacity (fBC) [mL]	0.240 ±0.070	0.120 ±0.012*	0.185 ±0.022**	0.262 ±0.098	*0.001 vs group I **0.002 vs group II
Detrusor index (DI) / detrusor overactivity index (DOI) [cm H_2O/mL]	121.9 ±33.0	625.8 ±101.4*	512.3 ±59.7**	132.4 ±42.1	*0.001 vs group I **0.004 vs group II
Motility index (MI) [cm H_2O × s/min]	185.4 ±45.9	245.2 ±61.5*	223.7 ±31.7**	203.7 ±31.0	*0.009 vs group I **0.005 vs group II

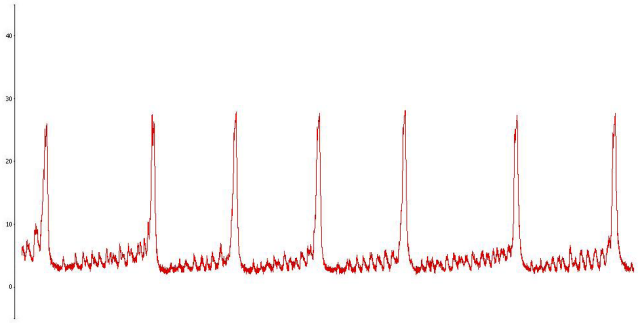
* $p < 0.05$ vs group I; ** $p < 0.05$ vs group II.

Results

The effect of intravesical hyperosmolar stimulation on urinary bladder activity in normal rats (group I and II)

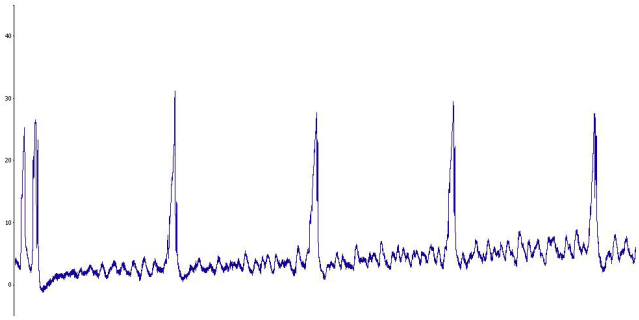
Intravesical infusion of hyperosmolar (2080 mOsm/L) saline solution induced DO. All hyperosmolar DO rats did not exhibit macroscopically signs of bladder inflammation, i.e. redness, oedema as well as wall thickening, mucosal erosions, ulcerations, petechial hemorrhages on the serosal surface. Cystometric evaluations revealed a significant decrease of intercontraction intervals (104%) and functional bladder capacity (100%). Additionally, an increase of basal pressure (118%), detrusor activity (413%) and motility index (33%) were observed (Table 1, Fig. 1, 2). No statistical differences of threshold, micturition voiding pressure and compliance were obtained.

Fig. 2. Cystometry in rats with hyperosmolar-induced DO



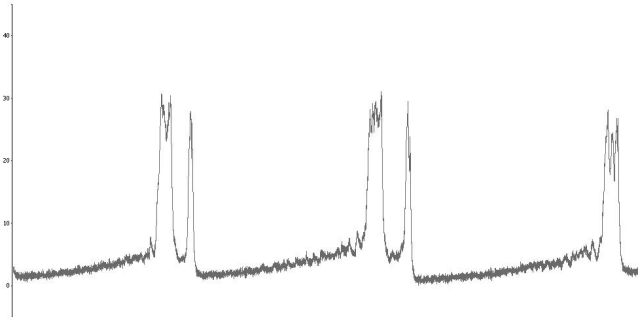
The figure shows 20-min interval – horizontal axis. Vertical axis estimates intravesical pressure of (-5) – 45 cm H_2O range.

Fig. 3. Cystometry in rats with hyperosmolar-induced DO after intravesical telmisartan administration



The figure shows 20-min interval – horizontal axis. Vertical axis estimates intravesical pressure of (-5) – 45 cm H_2O range.

Fig. 4. Cystometry in control rats after intravesical telmisartan administration



The figure shows 20-min interval – horizontal axis. Vertical axis estimates intravesical pressure of (-5) – 45 cm H_2O range.

The effect of intravesical administration of telmisartan on urinary bladder activity in rats with hyperosmolar-induced DO and in healthy rats (group III and IV)

The intravesical blockage of angiotensin II receptor type 1 by telmisartan diminished the severity of hyperosmolar-induced detrusor overactivity (Table 1, Fig. 3). In comparison with hyperosmolar-induced DO rats we observed a statistically significant increase of intercontraction interval (55%), functional bladder capacity (54%), compliance (140%). Also, a statistically significant decrease of detrusor overactivity index (18%) and motility index (9%) were observed. The difference of basal, threshold and micturition voiding pressure were not statistically significant. On the other hand, the intravesical blockage of angiotensin II receptor type 1 by telmisartan has no significant impact on urinary bladder function in naïve rats (Table 1, Fig. 4).

Discussion

Clinical evidence of hypertensive patients with lower urinary tract symptoms (LUTS) treated with different types of antihypertensive drugs shows that patients taking angiotensin II receptor blockers (ARBs) report a reduction of LUTS severity as compared to others on angiotensin-converting enzyme inhibitors or calcium channel blockers.[12] This fact suggests that angiotensin II receptors play a role in urinary bladder function. The pathophysiological role of angiotensin II in the cardio-vascular system was established in detail. Angiotensin II regulates the vascular tone and smooth muscle cells growth, as well as stimulating collagen production via AT1 receptors.[13] The data about its function in lower urinary tracts is still sparse. The function of AT1 receptors in detrusor overactivity development remains unclear. So far there is no research on the importance of angiotensin II in bladder dysfunction in humans. Few repots exist showing that the modulation of renin-angiotensin-aldosteron activity affects the lower urinary tracts function. Ito et al.[12] indicate that the International Prostate Symptom Score (IPSS) describing lower urinary tract symptoms (LUTS) is lower in patients with arterial hypertension treated with angiotensin II receptor blockers. On the other hand, Elliott et al.[14] described that angiotensin converting enzyme (ACE) inhibitors and ARBs are associated with the reduction of urge urinary incontinence especially in men. There are several animal models establishing the role of angiotensin II mainly in the course of bladder out-

let obstruction, hypertensive rats or estrogen-deficient ovariectomized animals. Our experiment revealed that the intravesical blockage of angiotensin II receptor type 1 by telmisartan diminished the severity of hyperosmolar-induced detrusor overactivity (DO). Therefore, besides the well-studied pathomechanisms of DO, a AT1-dependent pathway seems to be at least partially involved in the pathogenesis of DO. Angiotensin II – dependent pathway seems to be involved in urinary bladder motor activity. Both angiotensin I and II induced a potent contraction of the human detrusor muscle. It is probable that angiotensin I is converted to angiotensin II by ACE in the detrusor muscle, and angiotensin II subsequently mediates detrusor contraction.[15] Angiotensin II participates in detrusor muscle cells growth and collagen production within urinary bladder wall. Cheng et al.[16] postulated some pathomechanisms responsible for detrusor motor activity and collagen changes in urinary bladder dysfunctions. An interesting study of Shimizu et al.[17] on spontaneously hypertensive rats showed that ACE inhibitors ameliorates urodynamic parameters and urinary bladder oxidative injury as compared to normal rats. Thus, telmisartan also may restore proper urinary bladder blood flow and consequently prevent from oxidative stress induction and bladder damage leading to OAB/DO. The current pharmacotherapy and intravesical botulinum toxin administration are well developed.[18–20] However, such therapy is not fully beneficial. Therefore, new treatment options are required. Taking into account all of the above mentioned facts the modulation of angiotensin II – dependent pathway may have an impact on more sufficient treatment of OAB/DO.

Conclusions

Detrusor overactivity, resulting from intravesical increased osmolarity, seems to be at least partially mediated by angiotensin II type 1 (AT1) receptors activity. Therefore, angiotensin II receptors dependent pathways may become a potential target for urinary bladder dysfunction treatment using angiotensin II receptors blockers, especially in patients with co-existing arterial hypertension.

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Application of Fourier transform infrared spectroscopy to biomolecular profiling of cultured fibroblast cells from Gaucher disease patients: A preliminary investigation

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D – writing the article; E – critical revision of the article; F – final approval of article

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Conflict of interest
None declared

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Abstract

Background. Gaucher disease (GD) is defined as an autosomal recessive disorder resulting from the deficiency of glucocerebrosidase (E.C. 3.2.1.45). Glucocerebrosidase is responsible for the degradation of glucosylceramide into ceramide and glucose. The deficiency of this enzyme results in the accumulation of undegraded glucosylceramide, almost exclusively in macrophages. With Fourier transform infrared (FTIR) spectroscopy, the complete molecular diversity of the samples can be studied comparatively and the amount of the particular materials can be determined. Also, the secondary structure ratios of proteins can be determined by analysing the amide peaks.

Objectives. The primary aim of this study is to introduce FTIR-ATR spectroscopy technique to GD research for the first time in the literature and to assess its potential as a new molecular method.

Material and methods. Primary fibroblast cell cultures obtained from biopsy samples were used, since this material is widely used for the diagnosis of GD. Intact cells were placed onto a FTIR-ATR crystal and dried by purging nitrogen gas. Spectra were recorded in the mid-infrared region between 4500-850 cm^{-1} wavenumbers. Each peak in the spectra was assigned to various organic biomolecules by comparing their wavenumbers with the reference values in the literature. A quantitative analysis was performed using peak areas and we also used a hierarchical cluster analysis as a multivariate spectral analysis.

Results. We obtained FTIR spectra of fibroblast samples and assigned the biomolecule origins of the peaks. We observed individual heterogeneity in FTIR spectra of GD fibroblast samples, confirming the well-known phenotypic heterogeneity in GD at the molecular level. Significant alterations in protein, lipid and carbohydrate levels related to the enzyme replacement therapy were also observed, which is also supported by cluster analysis.

Conclusions. Our results showed that the application of FTIR spectroscopy to GD research deserves more attention and detailed studies with an increased sample size in order to evaluate its potential in the diagnosis and follow-up of GD patients.

Key words: FTIR, Gaucher disease, fibroblast

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Gaucher disease (GD) is the most frequent lysosomal glycolipid storage disorder, defined as an autosomal recessive disease resulting from the deficiency of glucocerebrosidase (GBA) (E.C.3.2.1.45), a lysosomal hydrolase, also known as acid-β-glucosidase. GBA is responsible for the degradation of glucosylceramide (GC), a natural glycosphingolipid, into ceramide and glucose. The deficiency of this enzyme results in the accumulation of undegraded glucosylceramide, almost exclusively in macrophages. GD is a multisystemic disorder, characterized by a spectrum of the clinical subtypes. GD is classically divided into non-neuronopathic type I (OMIM #230800) and neuronopathic types II (OMIM #230900) and III (OMIM #231000) variants. Although the most symptomatic individuals have hematological, visceral and skeletal diseases, there is a wide variation in the pattern and severity of extra-neurological involvement among these patients. Enzyme replacement therapy (ERT) is the preferred strategy in the treatment of GD.[1–3]

Infrared (IR) radiation is a noninvasive and nondestructive type of radiation and it causes the vibration of the covalent bonds of molecules when absorbed by tissues, body fluids or cells. Fourier transform infrared (FTIR) spectroscopy is a widely-used and preferred method of IR spectroscopy due to its speed and sensitivity. The resulting peaks correspond to the frequencies of vibrations between the bonds of the atoms making up the sample.[4,5] Different functional groups absorb characteristic frequencies and the resulting spectrum represents the unique molecular fingerprint of the sample material.[6] Attenuated total reflectance (ATR) is one of the approaches for FTIR sampling, which allows us to measure the analyte directly by using the ATR crystal. With FTIR, the complete molecular diversity of the samples (all types of organic and many types of inorganic compounds) can be studied comparatively with the knowledge of the peak origins, such as lipids, proteins, carbohydrates and nucleic acids. The amount of the particular compounds can also be determined by this method. Additionally, the secondary structures of proteins that are important for understanding the molecular mechanisms underlying the diseases can be determined by comparing the second derivatives of amide peaks (mainly Amide I peak) in FTIR spectrum that originate from proteins.[7–12]

The gold standard for the diagnosis of GD is the measurement of β-GBA activity in leukocytes or cultured fibroblasts obtained from the patients by skin punch biopsy.[3] The primary aim of this preliminary study is to introduce and evaluate the potential of ATR-FTIR spectroscopy technique for biomolecular profiling of cultured fibroblast cells derived from GD patients, as well as to investigate the individual phenotypic heterogeneity of the disease at the molecular level.

Material and methods

Patients and sample collection

Study group consisted of 8 unrelated Turkish GD patients representing 5 different genotypes who were selected from among the patients admitted to Hacettepe University, Department of Pediatric Gastroenterology (Ankara, Turkey). There were also 2 healthy adult controls. The diagnosis was suspected on account of the clinical features, which included the age of onset, organomegaly, skeletal features, presence of progressive central nervous system symptoms, as well as reduced cellular GBA activity. Fibroblast cells were obtained from the patients by 0.4 cm skin punch biopsy from the forearm under sterile conditions, following their written informed consent, with the approval of the institutional ethical review board. Clinical features of GD patients are presented in Table 1.

Fibroblast cell culture and sample preparation for FTIR spectroscopy

For FTIR studies, primary skin fibroblast cell culture samples were used. The biopsy samples were washed twice with phosphate buffered saline (PBS) containing 1% fetal calf serum (FCS, Biochrome AG, Berlin, Germany), 3% penicillin/streptomycin (Biochrome AG) and 3% amphotericin B solution (Biochrome AG). The biopsies were placed in 60 mm tissue culture dishes and minced. The small pieces were adhered onto the dishes with the help of a lancet and supplemented with 20% fetal bovine serum (FBS, Biochrome AG), 1% penicillin-streptomycin and 2 mM L-glutamine (Biochrome AG) in high glucose (4.5 mg/ml) DMEM (Biochrome AG). The cells were maintained in high glucose DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 2 mM L-glutamine in a humidified atmosphere containing 5% CO_2 at 37°C. A 75 cm^2 culture flask of fibroblast (90–95% confluency) was washed with 1X PBS for 3–4 times and the fibroblasts were detached by mechanical scraping and counted. For the standardization of the spectroscopic measurements, after the total number of the detached cells was set to 5×10^6, each tube was centrifuged at 1000 × g and the cell pellets were resuspended in 300 µl of 1X PBS for FTIR spectroscopy studies.

FTIR spectroscopy data collection and evaluation

Infrared spectra were obtained by a Bruker Tensor 27 FTIR (Bruker Optik GmbH, Ettlingen, Germany) equipped with a liquid N_2 cooled photovoltaic Mercury Cadmium Telluride detector and a universal ATR cell with a ZnSe crystal (Pike Technologies, Wisconsin, U.S.A). Continuously purging N_2 gas was provided during all the measurements. Samples were measured after hav-

Table 1. Clinical information about the patients included in this study

Patient No.	Diagnosis	Mutation	Age of diagnosis	Period of ERT
G1	Type I (M)	N370S/-	4 years	6 years
G2	Type III (S)	D409H/D409H	21 months	17 months
G3	Type I (M)	N370S/-	21 months	–
G4	Type I (+ FMF) (M)	N370S/-	10 years	8 months
G5	Type III (S)	L444P/L444P	8 months	–
G6	Type III (S)	L444P/L444P	9 months	–
G7	Type I (S)	N370/RecAHI	2 years	–
G8	Type I (S)	L296V/L296V	3 years	3 years

M – moderate; S – severe; ERT – enzyme replacement therapy; FMF – Familial Mediterranean Fever.

ing been dried on the crystal, similar to the method applied in the literature.[14,15] Briefly, fibroblast cell samples were mixed gently prior to the measurements to obtain homogeneity. 2.5 µL of cell suspension in PBS were spotted onto the ZnSe ATR crystal. The sample was dried on the crystal by evaporation using a mild N_2 gas stream for 3 min. PBS without cells was also measured under the same conditions and used as a background spectrum for subtraction. The air was recorded and subtracted automatically by the software before all the measurements. The spectra recorded in the mid-infrared region, between 4500–850 cm^{-1} wavenumbers and interferograms were accumulated for 50 scans at 4 cm^{-1} resolution at 22°C in the single-bounce ATR mode. Each sample was measured 3 times, the spectra were compared if they were identical and the average spectra were used for further analyses. All the data collection, manipulation and analyses were performed by OPUS software (Bruker Optik GmbH, Ettlingen, Germany).

PBS spectrum was subtracted from the sample spectra by using the OPUS software. The difference spectra were baselined using the rubber-band method and the resulting baselined spectra were used for the exact integration calculations. Min–max normalization was applied with respect to the Amide A (between 3500–3100 cm^{-1}) and Amide I peaks (between 1700–1600 cm^{-1}) for the regions between 3500–2750 and 1800–850 cm^{-1} wavenumbers, respectively for illustrative purposes. The second derivatives of the absorbance spectra, in which the absorption maxima appear as the minima, were calculated using Savitzky-Golay algorithm with 9 smoothing points. For comparing protein secondary structures, relative intensity values of the second derivative peaks in the Amide I region (1700–1600 cm^{-1}) were used; they were obtained by automated peak picking after vector normalization.

In order to achieve spectral differentiation, according to the molecular fingerprints of the samples, a cluster analysis (hierarchical clustering) was carried out using the OPUS software. Ward's algorithm with Euclidian distances was used to construct the dendograms, which is known to give good predictions among the available algorithms.[16,17] Sensitivity and specificity values were calculated for cluster dendrograms as described by Severcan et al.[17]

Statistics

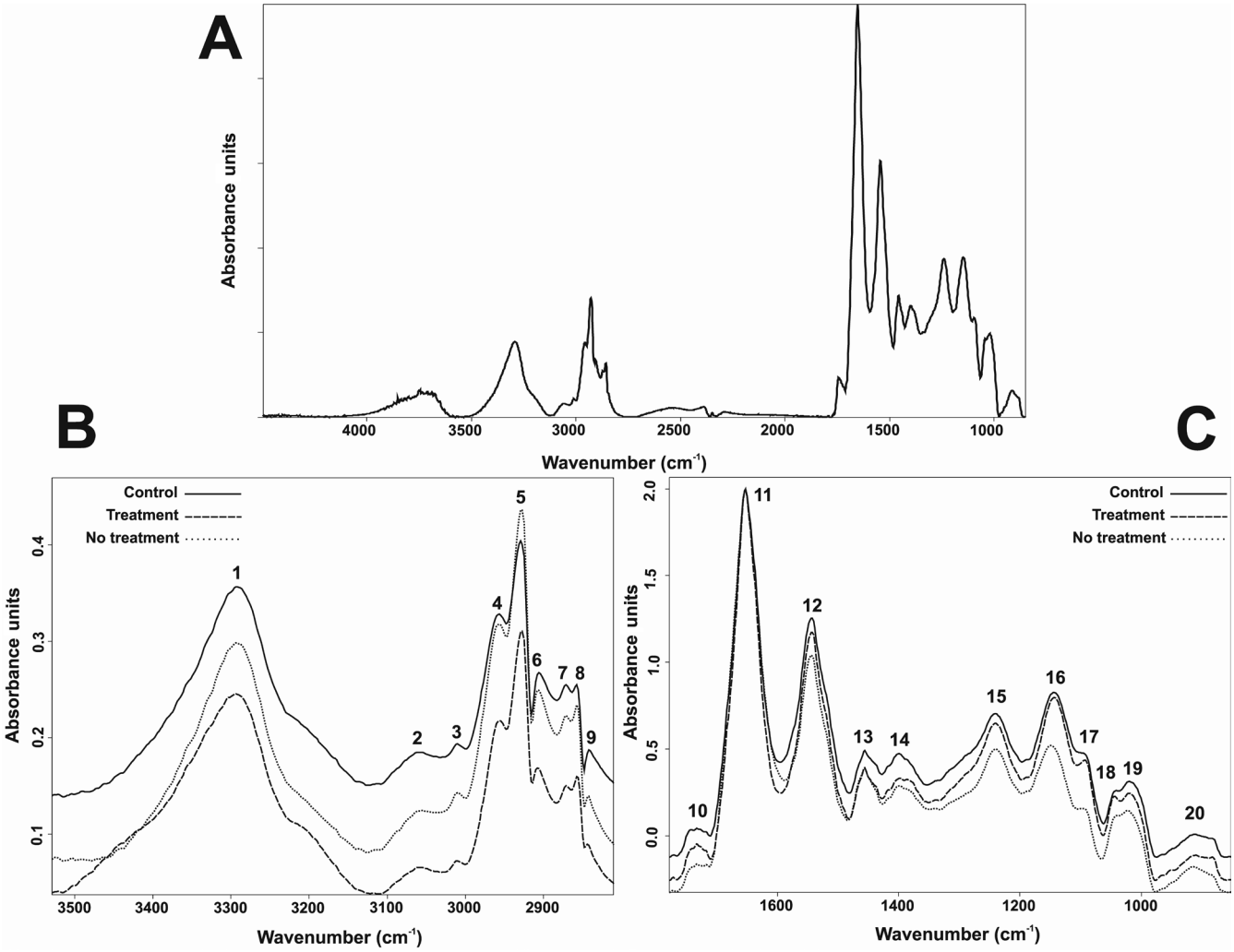
The differences of the means were compared by Student's t-test. Levene's test for homogeneity of variances was performed prior to the statistical tests and Levene's p-values were taken into consideration while evaluating the results of Student's t-test. The p-values equal or less than 0.05 were considered as significantly different and the results were expressed as means ±SD. SPSS 13.0 software was used for the analyses.

Results

Biomolecular characterization of the fibroblast cells based on the FTIR spectrum

We obtained FTIR spectra of the fibroblast cell samples between 4500–850 cm^{-1} wavenumbers in the mid-infrared region. The spectra were complex with many characteristic peaks. Fig. 1A shows the representative FTIR absorbance spectrum obtained by averaging all the spectra used in the analyses. According to our results, there are 2 main regions in the FTIR spectra of GD and control fibroblast cell samples: the region between 3500–2750 cm^{-1} (Fig. 1B), which can be considered as a lipid dominated region (except some contribution from proteins), and 1800–850 cm^{-1} (Fig. 1C), which is referred to as a fingerprint region.[18–20] We calculated the total lipid content as a sum of the areas of the peaks 2, 3, 4, 5, 6, 8, 9 and 13, which originate from lipids. The band between 4100–3580 cm^{-1}

Fig. 1. Representative FTIR absorbance spectrum



A – between 4500–850 cm^{-1} obtained by averaging all the spectra used in the analyses; B – between 3500–2750 cm^{-1}; C – 1800–850 cm^{-1} wavenumbers. Refer to Table 2 for spectral assignments of numbered peaks. The stacked spectra show aletrations between mean spectra of control, treatment and no-treatment samples, and were normalized with respect to the Amide I peak between 1700–1600 cm^{-1} after baseline correction.

was ignored, since it was originated from O–H stretchings of residual H_2O.[21] Twenty major absorption peaks in our FTIR spectra, which are numbered in Fig. 1, were assigned in the present study to various biomolecules such as proteins, lipids, cholesterol esters, nucleic acids and carbohydrates (Table 2) by matching the wavenumbers with the references obtained from the literature (references are cited in the caption for Table 2).

Peaks at 3294 (Amide A), 3060 (Amide B), 2871 (CH_3 symmetric stretching), 1653 (Amide I) and 1544cm^{-1} (Amide II) wavenumbers mainly originate from various bond vibrations of proteins (Table 2). Region between 3500–2750 cm^{-1} wavenumbers contains contribution mainly from lipids. CH_3 asymmetric stretching (2957 cm^{-1}), CH_2 asymmetric (2929 cm^{-1}) and symmetric (2857 cm^{-1}) stretching, olefinic =CH (3011 cm^{-1}) and C–H (2843 cm^{-1}) stretching modes are the major bond vibrations in this region, originating from lipids (Table 2). Also, there is a peak at 1455 cm^{-1} that was assigned to CH_2 bending of lipids. Saturated ester C=O stretching at 1732 cm^{-1} originates from the cholesterol esters or phospholipids. Peaks between 1093–914 cm^{-1} wavenumbers mainly result from phosphate groups of nucleic acids, phospholipids and phosphorylated proteins and C–O stretching of carbohydrates.

Comparative analysis of the levels of biomolecules

The intensity or area of a particular FTIR absorption peak reflects the amount of the assigned compound for that wavenumber.[12,19] Although the primary purpose of this preliminary study is to introduce the method to GD research rather than to understand the molecular mechanisms underlying GD, we also included the control group in order to see if there were very prominent differences. First of all, the quantitative and qualitative comparisons were performed between GD and control groups in terms of spectral differences. We observed overlapping peaks

Table 2. Peak assignments of major absorbance peaks of fibroblast samples detected in the present study and their definition with relative organic compounds based on the literature[6,12,17–19,22–26] (peak numbers illustrated in Fig. 1B, C)

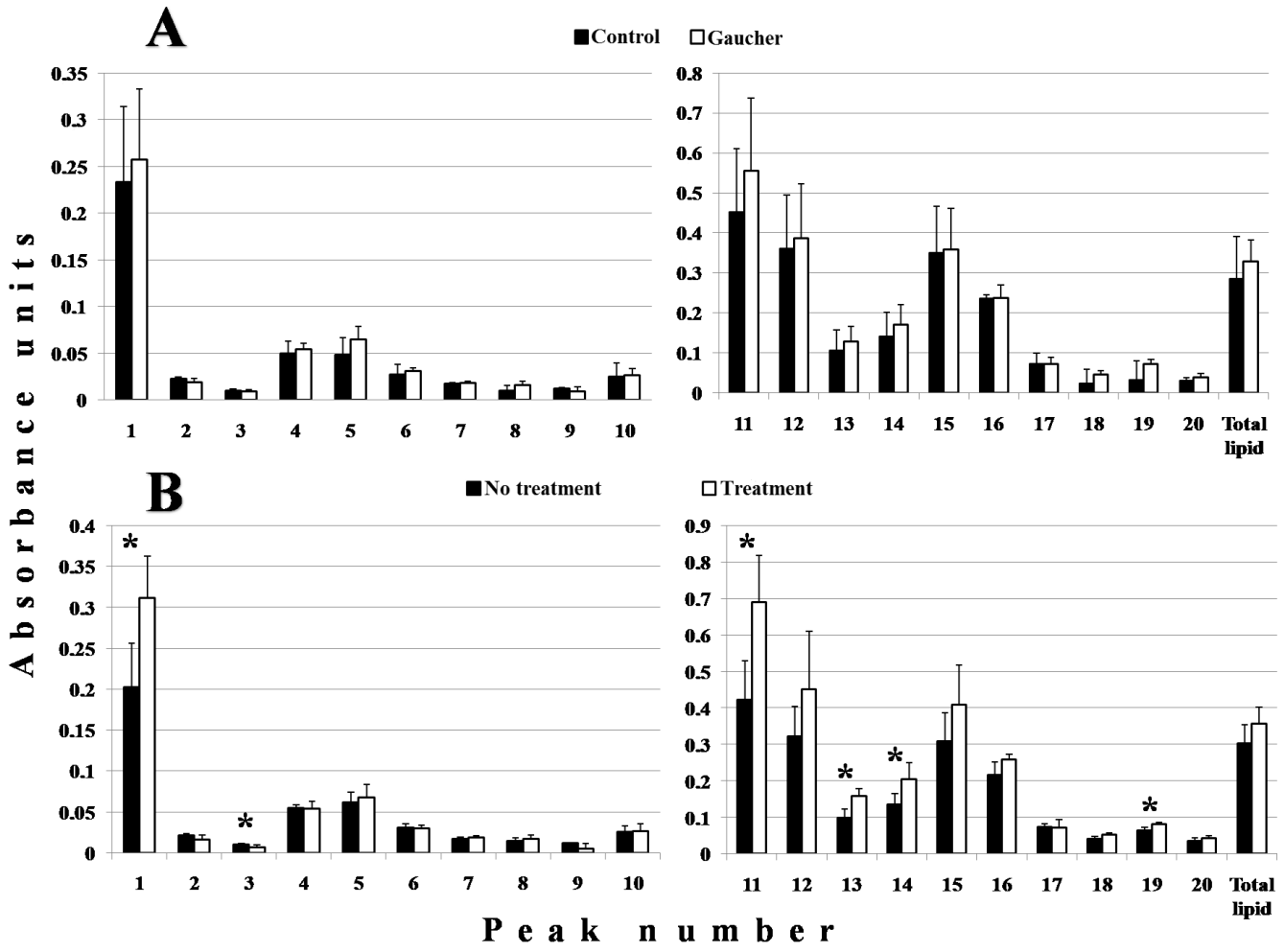
Peak No.	Wavenumber (cm^{-1})	Definition	Organic compound
1	3294	Amide A; mainly N–H stretching of proteins with contribution from intermolecular H bondings and O–H stretching mode of polysaccharides	mainly proteins
2	3060	Amide B; C–N, N–H stretching of proteins	proteins
3	3011	olefinic, C–H stretching mode of the HC=CH groups	unsaturated lipids
4	2957	CH_3 antisymmetric stretching	mainly lipids
5	2929	CH_2 antisymmetric stretching	mainly lipids
6	2907	CH_2 and CH_3 stretching of phospholipids, cholesterol and creatine	mainly lipids
7	2871	CH_3 symmetric stretching	mainly proteins
8	2858	CH_2 symmetric stretching	mainly lipids
9	2843	C–H stretching	mainly lipids
10	1732	Saturated ester C=O stretching	cholesterol esters, phospholipids, ester functional groups in lipids
11	1653	Amide I; 80% C=O stretching, 10% N–H bending, 10% C–N stretching	proteins
12	1544	Amide II; 60% N–H bending, 40% C–N stretching	proteins
13	1455	CH_2 bending	lipids
14	1396	COO– symmetric stretching	fatty acids
15	1241	PO_2^- antisymmetric stretching (non H-bonded)	nucleic acids, phophorylated proteins and phospholipids
16	1147	C–O stretching	carbohydrates/glycogen, nucleic acids
17	1094	PO_2^- ionized symmetric stretching (fully H-bonded) of phosphodiester groups, C–O stretcing	nucleic acids, phospholipids, glycogen, oligosaccharides and glycolipids
18	1041	C–O stretching, coupled with C–O bending of the C–OH groups of carbohydrates	oligosaccharides, polysaccharides
19	1021	C–O stretching, coupled with C–O bending of the C–OH groups of carbohydrates	oligosaccharides, polysaccharides
20	976–875	$C–N^+–C$ stretching	nucleic acids (DNA, RNA), ribose-phosphate main chain vibrations of RNA, phosphate monoesters

of GD and control samples and detected some quantitative differences and minor peak shifts between GD and control group, as shown in Fig. 1B, C. Means of absorbance peak area values of GD and control groups obtained by integration are presented as bar graphics in Fig. 2A. According to our results, lipid, protein and carbohydrate levels were increased in GD when compared to controls.

Moreover, we grouped GD samples according to the severity (severe or moderate), type (Type I, II and III) and treatment (ERT) condition (Table 1). We observed statistically significant differences between the samples of patients who had been taking ERT treatment and those who had not received treatment. The areas of the peaks at 3294, 1653, 1455, 1396 and 1021 cm^{-1} wavenumbers indicating lipids, proteins and polysaccharides increased in the samples after treatment (Fig. 1B, 1C, 2B). However, we did not observe a significant decrease in the areas of the peaks originating from CH_2 and CH_3 anti-symmetric and symmetric stretching, which are the main IR absorption peaks of lipid backbone, including acyl chains of GC.

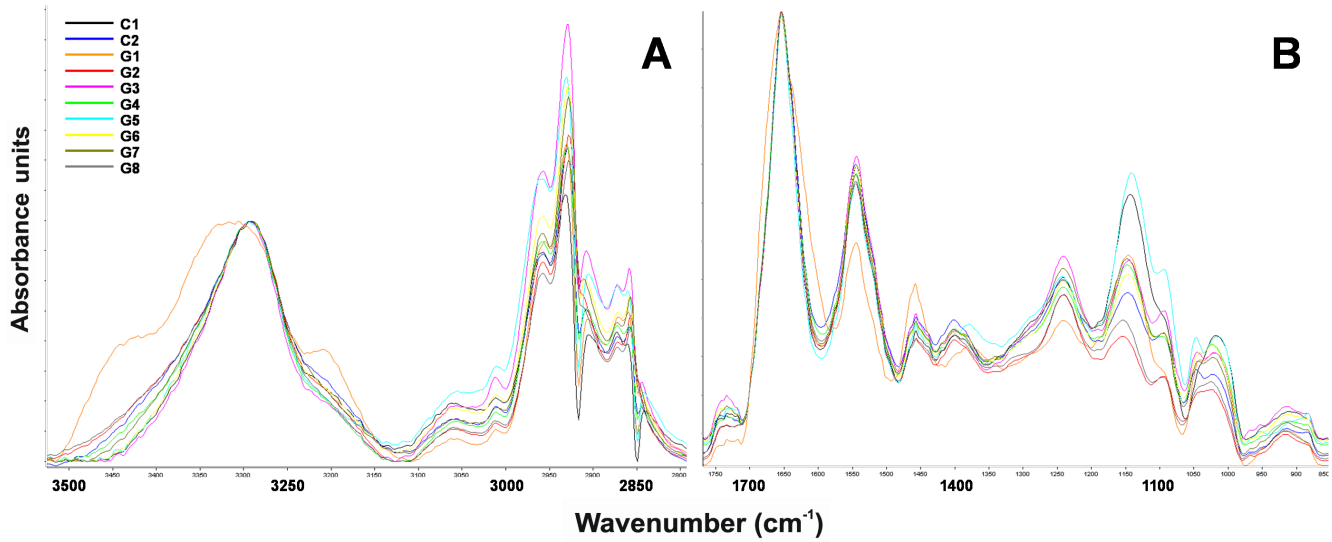
Since one of the aims of the present study is to investigate the phenotypic heterogeneity of GD fibroblast cells by FTIR, we also analyzed the individual variation regarding the peak patterns of the samples. We observed some differences between the spectra in terms of peak area and shifts (Fig. 3).

Fig. 2. Demonstration of mean values (±SEM) of baselined absorbance band areas



A – GD and control; B – treatment and no-treatment groups based on our results. Peak numbers are illustrated in Fig. 1B, C and spectral assignments are given in Table 2.

Fig. 3. Individual variation in the absorbance spectra of GD patients (G1–G8) and controls (C1, C2)



A – between 3500–2750 cm^{-1}; B – between 1800–850 cm^{-1} wavenumbers. The spectral range between 3500-2750 cm^{-1} and 1800-850 cm^{-1} were normalized with respect to the Amide A band between 3500–3100 cm^{-1} and Amid I band between 1700–1600 cm^{-1}, respectively after baseline correction.

Comparative analysis of the protein secondary structures

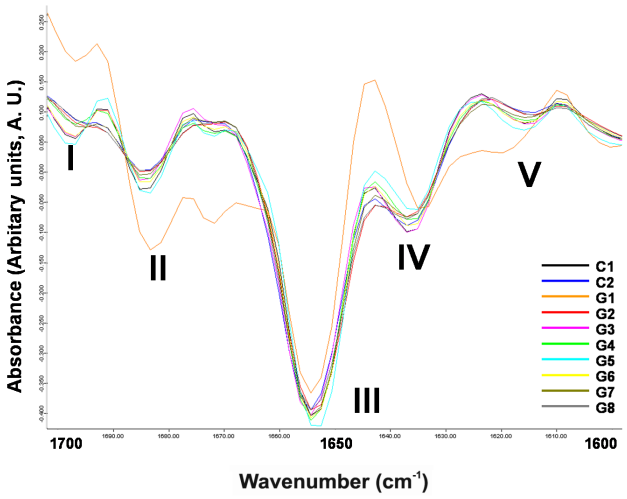
FTIR peaks, including Amide I, consist of several sub-peaks, which can be obtained by derivatization.[19] We analyzed the second derivative spectra of Amide I peaks (1653 cm^{-1}) in order to determine and compare protein secondary structures, which is very important in biological systems.[9,23] Amide I peak is sensitive to protein conformational changes and is usually used to determine protein secondary structures based on FTIR spectra.[7–11] According to our results, the second derivative of the Amide I peak had 5 sub-peaks at 1697, 1684, 1654, 1636 and 1615 cm^{-1} (Fig. 4). Peak assignments for protein secondary structures are given in Table 3. We observed slight differences and peak shifts between the control and GD groups (Table 4). α-helix structure was observed as being slightly increased in GD, while β-sheet structure was decreased. These structures decreased slightly in samples after treatment, compared to no-treatment group. Statistical tests did not reveal significant differences between GD-control, type I-II-III, severe-moderate and with-without treatment groups in terms of peak intensities and shifts.

Also, we investigated the individual variations in the second derivative peak patterns of the Amide I peak (Fig. 4). We observed individual alterations on protein secondary structures in terms of peak intensity and frequency shifts.

Table 3. Peak assignments for secondary derivative spectrum of Amide I peak indicating protein secondary structures based on the literature.[6–8,10,11] See Fig. 4 for peak numbers

Peak No.	Wavenumber (cm^{-1})	Protein secondary structure
I	1697	aggregated strand
II	1684	antiparallel β-sheet, β-turns
III	1654	α-helix
IV	1636	β-sheet
V	1615	amino acid side chain vibrations, intermolecular β-sheets, aggregated strand

Fig. 4. Second derivative spectra of Amide I absorption peak of the samples between 1700–1600 cm^{-1} wavenumbers (with a center at 1653 cm^{-1}), which demonstrates main protein secondary structures and individual variation. Absorption maxima appear as minima and the spectra were vector normalized. Refer to Table 3 for peak numbers and their assignments



Classification by hierarchical cluster analysis

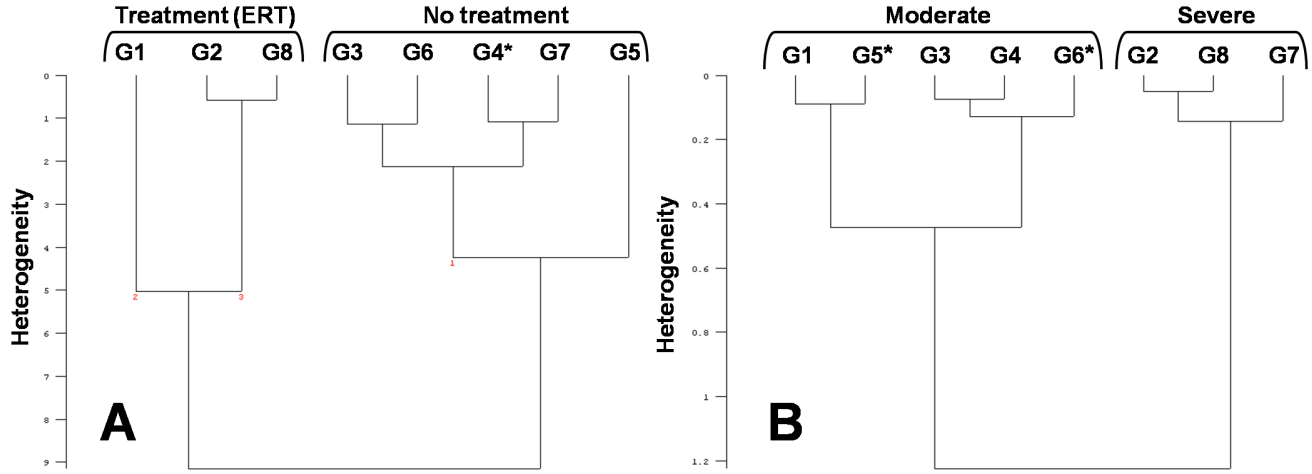
We performed hierarchical cluster analysis in order to achieve spectral differentiation according to the molecular fingerprints of the samples. The resulting dendrograms were evaluated in regard to such clinical features as severity, type of the disease, mutation and treatment condition. Our analyses yielded a classification between the sample of patients who had been taking ERT treatment and those who were not at the time of sampling, based on the 2 main spectrum areas (lipid and fingerprint regions) of the min–max normalized spectra (Fig. 5A).

In the present study, we also observed partial differentiation within GD group according to the severity of the disease, but it was not as significant as the obtained classification related to the treatment. Using the region between 3035–2820 cm^{-1} after the second derivative and vector normalization preprocessings, we obtained a dendrogram which partially classified GD patients into severe and moderate groups (Fig. 5B).

Table 4. Means of vector normalized second derivative relative peak intensities in Amide I region of GD and control groups. Relative intensity values obtained after peak picking by OPUS software. See Fig. 4 for peak numbers and Table 3 for their spectral assignments

Peak No.	Gaucher disease	Control	GD before treatment	GD after treatment
I	0.014 ±0.015	0.015 ±0.021	0.024 ±0.016	0.004 ±0.005
II	0.095 ±0.026	0.089 ±0.008	0.087 ±0.007	0.104 ±0.037
III	0.435 ±0.033	0.428 ±0.001	0.450 ±0.017	0.420 ±0.040
IV	0.076 ±0.018	0.085 ±0.012	0.078 ±0.016	0.074 ±0.022
V	0.013 ±0.007	0.012 ±0.012	0.015 ±0.007	0.011 ±0.007

Fig. 5. Representation of a cluster analysis dendrogram



A – the samples of patients with or without treatment; B – the samples of patients diagnosed as severe or moderate; * – misgrouped samples; sensitivity and specificity are 75% and 100% for treatment group, 100% and 75% for no-treatment group classification whereas these values are 60% and 100% for severe and 100% and 60% for moderate group classifications, respectively.

Discussion

Our optimized procedure allowed us to characterize the major biomolecules in the cultured fibroblast cells of GD patients in a qualitative and quantitative manner. According to our results, lipid, protein and carbohydrate levels were found to be increased in GD compared to controls. GC (a glycosphingolipid) is the major GD-related lipid type that accumulates in the cells with GD. Secondly, glucosysphingosine is also accumulated by GD cells but at much lower concentrations.[27] A combined increase in the lipid and carbohydrate levels may reflect increased GC storage in lysosomes, as a result of the deficiency of GBA in GD patients. Since GD is a rare disease, our sample size is enough for a preliminary study aiming to introduce the method. We observed some significant molecular alterations (e.g., lipid, protein and carbohydrate levels) between groups with and without treatment. A comparative follow-up study with a larger sample size of GD and controls should be done in order to show correlations between the biomolecule levels (especially GC) and GD condition.

Moreover, our analyses on the spectral patterns revealed that GD fibroblast samples showed an individual variation at the molecular level. These alterations were mainly in protein, carbohydrate and fatty acid levels and structures, according to our results. More than 250 GD-related mutations in the GBA gene have been identified to date.[2] Emre et al. reported that L444P and N370S were the most prevalent mutations among Turkish GD patients.[13] According to the literature, genotype-phenotype correlation is not seen in GD and clinical symptoms show heterogeneity.[1,2,13] The results of the present study also showed no correlation between the genotype and the molecular fingerprint of GD fibroblast samples, confirming the diverse phenotypic expression and heterogeneity in GD. Additionally, our results showed that phenotypic variation must be taken into consideration in FTIR spectroscopic studies on GD and the sample size must be accordingly large to eliminate individual variations.

Our results have also indicated conformational changes in proteins between experiment groups. However, our small sample size did not allow us to perform comprehensive statistical tests for detecting possible significant correlations. Since the main purpose of this preliminary study was to introduce FTIR spectroscopy to GD research, rather than to reveal the molecular mechanisms of the disease, our results would still be useful for the method optimization as a reference work. Our results also showed that GD fibroblast samples expressed phenotypic heterogeneity in terms of the second derivative peak pattern reflecting the change in protein conformation.

Our preliminary multivariate cluster analysis yielded a spectral classification of the groups with and without treatment, suggesting that the treatment process of GD may cause alterations in the level of biomolecules in GD fibroblasts. In our study, only 1 sample with treatment (G4) was classified in no-treatment group. This misgrouping may be related to the duration of the treatment, since G4 patients had the shortest treatment period, compared to other patients. Our hierarchical cluster analysis results supported our statistical tests made by using individual peak areas. The classification regarding the severity of the disease was less significant. G5 and G6 patients were clinically diagnosed as severe but classified into the moderate group in the dendrogram (Fig. 5B), posssibly as a result of the phenotypic heterogeneity of the samples.

As a summary, primary skin fibroblast cell culture samples obtained from GD patients were studied by using FTIR-ATR for the first time to introduce the technique

and to assess the potential of molecular IR fingerprints as an alternative approach in research related to lysosomal glycolipid storage disorders. We assigned different peaks of the complex FTIR spectrum to various bioorganic molecules. We observed significant differences between the groups with or without treatment, indicating structural and functional alterations due to the ERT. Also, we observed individual variations reflecting the phenotypic heterogeneity of GD at the molecular level.

Our results showed that FTIR spectroscopy was a rapid and sensitive technique and had the ability to give detailed information about important functional groups of bioorganic molecules in fibroblast samples that are widely used for the diagnosis of GD. Our study also showed that this research tool might provide molecular insights into the pathophysiology of GD. FTIR spectroscopy has already been used in medical diagnostics for some diseases as an alternative to well-known biochemical and histochemical methods and it is suitable for routine use.[28,29] The present study was a complementary investigation to our ongoing proteomic analyses of GD and allowed us to carry our reserarch from the proteomic level to metabolomics in the biology systems frame. We attempted to introduce and apply FTIR spectroscopy to make molecular characterization of GD and our results showed that the application of this technique in GD research deserves further investigation to assess its potential in the diagnosis and monitoring of GD. Therefore, detailed studies should be done with an increased sample size.

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Histopathological evaluation of the effect of locally administered strontium on healing time in mandibular fractures: An experimental study

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Mandibular fractures are the most common facial fractures. They can be treated by conservative techniques or by surgery. The authors hypothesized that the application of a single local dose of strontium chloride would accelerate the healing of subcondylar mandibular fractures, shorten the recovery time and prevent complications.

Objectives. The aim of the present pilot study was to evaluate the effects of a single local dose of strontium chloride on the healing of subcondylar mandibular fractures in rats.

Material and methods. This randomized experimental study was carried out on 24 male Wistar albino rats. The rats were randomly divided into 3 groups: experimental group 1, receiving 3% strontium chloride; experimental group 2, receiving 5% strontium chloride; and the control group. A full thickness surgical osteotomy was created in the subcondylar area. A single dose of strontium solution (0.3 cc/site) was administered locally by injection on the bone surfaces of the fracture line created. Nothing was administered to the control group. The mandibles were dissected on postoperative day 21. The fractured hemimandibles were submitted to histopathological examination.

Results. The median bone fracture healing score was 9 (range: 7–9) in experimental group 1; 8 (range: 7–10) in experimental group 2; and 7.50 (range: 7–8) in the control group. When the groups were compared in terms of bone healing scores, there was a statistically significant difference between experimental group 1 and the control group ($p < 0.05$).

Conclusions. This study is the first to show that local strontium may have positive effects on the healing of subcondylar mandibular fractures. In the authors' opinion, 3% strontium was beneficial for accelerating facial skeleton consolidation and bone regeneration in rat subcondylar mandibular fractures. This treatment procedure may be combined with closed fracture treatment or a conservative approach.

Key words: strontium chloride, fracture healing, subcondylar mandibular fractures

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Mandibular fractures are the most common facial fractures in facial trauma and account for 23–97% of all facial fractures.[1] In the mandible and maxillofacial region, fractures of the mandibular condylar processes, which account for 25–35% of all mandibular fractures, are the most common fractures.[2]

Re-establishing anatomy, providing stabilization and restoring functionality with the least morbidity are the therapeutic targets of condylar fracture management.[3] These fractures can be treated using a conservative technique (closed reduction) or by surgery.[2,4,5] Generally, in the treatment of subcondylar mandibular fractures, external fixation with arch bars is performed for 3 weeks in non-displaced fractures (except when open reduction and internal fixation is indicated) and 6 weeks in displaced fractures.

Closed treatment requires a period of maxillomandibular fixation, followed by active physiotherapy. The closed reduction method may lead to complications such as pain, arthritis, an open bite, deviation of the mandible on opening, inadequate restoration of the vertical height of the ramus leading to malocclusion, and ankyloses in the long term.

Strontium salts are elements acting as secondary messengers and competing with calcium in the organism. Strontium is structurally similar to calcium and helps build bone. The positive effect of strontium on bone formation has been known since 1950.[6] Systemic administration of strontium has been reported to have a positive effect on bone healing by increasing osteoblastic activity and decreasing osteoclastic activity.[7]

The aim of the present pilot study was to evaluate the effects of a single local dose of strontium chloride on the healing of subcondylar mandibular fractures in rats. The authors hypothesized that applying a single local dose of 3% or 5% strontium chloride (SC) would accelerate the healing of subcondylar mandibular fractures, shorten the recovery time and prevent complications.

Material and methods

The experimental animals

The study protocol was approved by the Institutional Review and Animal Ethics Committee of the Cumhuriyet University School of Medicine (Sivas, Turkey), and the study was conducted according to "Guide for the Care and Use of Laboratory Animals".[8]

In this study, a randomized experimental protocol was used. The study was carried out on 24 male Wistar albino rats, aged 16–18 weeks, with an average body weight of 230 ±10 g. The rats were randomly divided into 3 groups: experimental group 1, receiving 3% SC (0.3 cc/site, n = 8); experimental group 2, receiving 5% SC (0.3 cc/site, n = 8); and the control group (n = 8).

The rats were housed in standard laboratory conditions (12 h light/dark cycles, 24 ±2°C, 35–60% humidity). Because of the broken jaws, all the animals were fed only soft food and water during the first 7 days of the experiment. The animals resumed normal diets (a standard laboratory diet and drinking water) after the 1st week.

The drug and chemicals

Strontium chloride was obtained from Sigma Chemical Co. (St. Louis, USA). This reagent was dissolved in saline and the purity of all the chemical reagents was at least analytical grade. The strontium solution (0.3 cc/site) was administered locally by injection on the bone surfaces of the fracture lines created.

Operation procedure and study protocol

The animals were anesthetized with intraperitoneal injections (7.5 mg/kg) of ketamine (Ketalar®, Pfizer Turkey, Istanbul, Turkey) and intramuscular injections (6 mg/kg) of xylazine (Rompun®, Bayer, Istanbul, Turkey). In each rat, the right buccal area was shaved and prepared with an antiseptic solution (povidone iodine). Following an approx. 10 mm incision made along the inferior border of the mandible and the division of the masseter muscle, a full thickness surgical osteotomy was created using mosquito forceps in the subcondylar area, which was confirmed by condyle fragment mobility. Hemostasis was induced both on the fracture line and the connected soft tissues, and a single dose of 3% SC (0.3 cc/site) was administered by injection to experimental group 1 (n = 8); and 5% SC (0.3 cc/site) was administered to experimental group 2 (n = 8). Nothing was administered to the control group (n = 8).

The wounds were not syringed and no debris was removed. Finally, the skin flaps were replaced and sutured. All the rats were administered intramuscular penicillin injections during the first 3 days after the operation.

After postoperative day 21, the animals were euthanized with intraperitonial injections of pentothal sodium (200 mg/kg). The mandibles were then dissected, all soft tissues were removed, and the fractured hemimandibles were submitted for histopathological examination.

Histopathological examinations

The histologic analyses were performed by 2 pathologists (HO, ET) who were blind to the samples. All the tissue examples were immediately fixed in 10% formalin. After fixation, the specimens were kept in 10% nitric acid. Decalcification was complete in 4 days and the specimens were embedded in paraffin. The specimens were cut in the sagittal sections into 5-µm-thick sections, transferred to slides for conventional hematoxylin-eosin (H&E) staining and examined by light microscopy (Eclipse 80İ, Nikon Instruments Inc., Tokyo, Japan). A digital cam-

Table 1. Histological scoring system for the evaluation of fracture healing

Score	Histological findings in the fracture zone
1 point	fibrous tissue
2 points	mainly fibrous tissue and small amounts of cartilage tissue
3 points	equal amounts of fibrous and cartilage tissue
4 points	entirely cartilage tissue
5 points	mainly cartilage tissue and small amounts of immature (woven) bone
6 points	equal amounts of cartilage tissue and immature bone
7 points	significant immature bone and small amounts of cartilage
8 points	entirely immature bone
9 points	immature bone and small amounts of mature bone
10 points	mature (lamellar) bone

era and auxiliary equipment (Nikon USB (H) EXT 1/0, Nikon Instruments Inc., Tokyo, Japan) were used with the microscope to obtain digital images of the sections.

The amount of the ossification for each section was scored on a grading scale ranging from 1 point (fibrous tissue) to 10 points (mature bone), as described by Huo and Troiano (Table 1).[9]

Statistical analysis

The data were analyzed using SPSS software for Windows (v. 22.0, SPSS Inc., Chicago, USA). Sections of all the specimens stained with hematoxylin-eosin were scored. The average scores were calculated for both groups and the differences between the groups were analyzed statistically. The data was expressed as mean, median and minimum-maximum. All of the group differences were analyzed with the Kruskal-Wallis test to investigate the cause of the discrepancy ($p < 0.05$) encountered with the Mann-Whitney U test.

Results

Three days after surgery, 2 rats in experimental group 1, 1 rat in the control group and 2 rats in experimental group 2 died due to dehydration and nutritional problems. Thus, no histological examination was performed on these rats. As a result, 6 rats in experimental group 1, 7 rats in experimental group 2, and 6 rats in the control group were included in the study. The application was well tolerated by all the rats and there was no significant weight loss until the day they were sacrificed.

Fig. 1. A histological section from the experimental group 1 treated with 3% SC; immature (woven) bone and small amounts of mature bone formation (score: 9; HE; ×200)

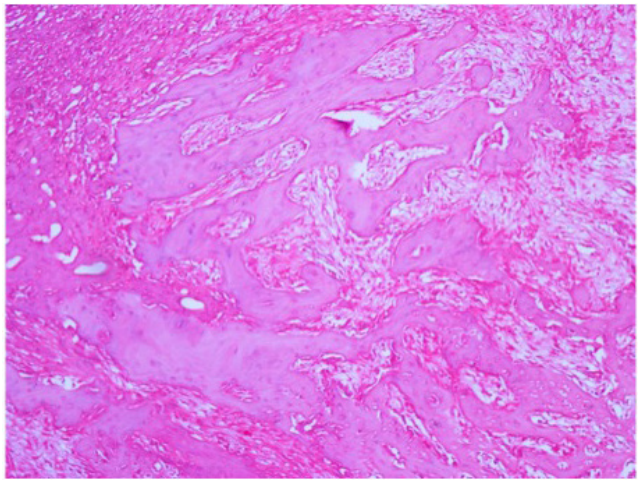
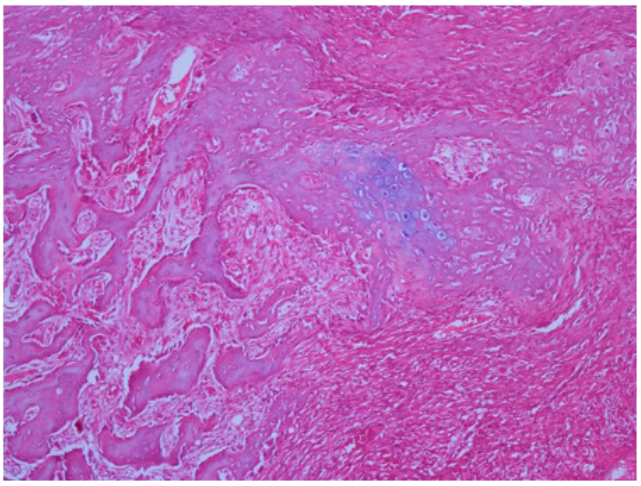


Fig. 2. A histological section from the control group; significant immature (woven) bone and small amounts of cartilage formation (score: 7; HE; ×100)



With regard to bone fracture healing, the histological scores of all the specimens stained with hematoxylin-eosin are given in Table 2. Histopathological examination revealed immature (woven) bone and small amounts of ma-

Table 2. Histological scores for fracture healing in all three groups

Groups	Min	Max	Median	Mode	Mean	p-value (KW)	p-value (MW)
Experimental group 1 (n = 6)	7	9	9	9	8.67 ±0.82	0.014*	1.000[a]
Experimental group 2 (n = 7)	7	10	8	10	8.57 ±1.40		0.234[b]
Control group (n = 6)	7	8	7.5	7	7.50 ±0.55		0.026[c]

* – differences in all groups were analyzed by the Kruskal-Wallis test; a – experimental groups 1 and 2 were analyzed by the Mann-Whitney U test; b – experimental group 2 and control group were analyzed by the Mann-Whitney U test; c – experimental group 1 and control group were analyzed by the Mann-Whitney U test.

ture bone formation in experimental group 1 (Fig. 1), and significant immature (woven) bone and small amounts of cartilage formation in the control group (Fig. 2).

The median bone fracture healing score was 9 (range: 7–9). There was immature bone and small amounts of mature bone in experimental group 1, with a median healing score of 9 (range: 7–9); completely immature (woven) bone in experimental group 2, with a median healing score of 8 (range: 7–10); and significant immature (woven) bone and small amounts of cartilage in the control group, with a median healing score of 7.50 (range: 7–8). When the groups were compared in terms of bone healing scores, there were no statistical differences between experimental groups 1 and 2 or between the experimental group 2 and the control group, while there was a statistically significant difference between the experimental group 1 and the control group ($p < 0.05$).

Discussion

This study was designed to investigate the impact of a single application of 3% and 5% SC on bone healing and the histologic characteristics of the new bone induced by these applications. The results showed a statistically significant difference between the controls and the rats treated with a single dose of 3% SC in terms of mean bone fracture healing score. It was remarkable that there were rats with mature (lamellar) bone formation in the group treated with 1 application of 5% SC. However, the controls and the group treated with 5% SC did not show any statistically significant difference in terms of bone healing scores.

Strontium, which has a strong affinity for hydroxyapatite, is an alkaline metal cation.[10] The similarity of strontium to calcium allows strontium to be incorporated into the mineral phase of bone. The positive effect of strontium on bone homeostasis was shown in some studies conducted in the 1950s.[6]

Awareness of the biological role of strontium has increased in recent years.[11,12] Physiological bone regeneration, a coordinated process, is maintained by bone-forming osteoblasts and bone-resorbing osteoclasts. The 2 factors produced by osteoblasts forming the bone: receptor activator NF-κB ligand (RANKL) and osteoprotegerin (OPG) control the activity and differentiation of osteoclasts.[14–16] The bone-building action of strontium works by down-regulating osteoclastogenesis, modulating the RANKL/OPG balancing system.[7,13] These effects of strontium have attracted the attention of many researchers, and many studies have been conducted on the local and systemic use of strontium. It has been reported that applying strontium systematically increases peri-implant bone volume and implant pullout ability in animal subjects with bone-integrated implants.[17,18] It has also been demonstrated that bioactive glass and glass-ceramics that include local strontium show biocompatibility.[19–22] The positive effects of strontium on peri-implant bone formation have led researchers to develop various methods of local strontium administration. These methods include strontium-substituted hydroxyapatite implants, modification of metal oxide layers with a strontium-containing salt, generation of strontium titanate nanotubes on the surfaces of metal implants, bioactive glass comprising strontium, and incorporation of strontium into metal oxide layers on implant surfaces.[23–27]

Strontium that is incorporated into bone cements has been found to improve bone formation and reduce bone resorption in vivo.[28] Sabareeswaran et al. investigated the bioactive fixation of strontium-containing glass ceramics and found out that it led to osseointegration during short-term implementation.[29]

Schumacher et al. investigated the effect of strontium(II) released from calcium phosphate bone cement on primary human mesenchymal stem cells and reported that it could be useful for local therapy, particularly in osteoporotic bone.[30]

Al-Duliamy et al. studied the effects of locally injected strontium on the anchoring unit of experimental relapsed tooth movement in a rat model. They noted that the strontium injections led to significant inhibition of both tooth movement and relapse movement, and that "histological examinations showed that strontium enhanced the number of osteoblasts and reduced the number of osteoclasts".[31]

Forsgren et al. showed that incorporating strontium into the surfaces of orthopedic and dental implants accelerated bone healing.[24] Hao et al. found that the use of strontium hydroxyapatite and alginate in an injectable in situ gel-forming system resulted in shorter healing times in a rat calvarial model compared with conventional graft surgery, and that the animals showed no signs of inflammation.[32] They also stated that this in situ gel-forming system could be an advantageous and minimally invasive process for local bone augmentation.

Conclusions

Treating subcondylar fractures is a controversial issue.[33] It can be conducted in 2 different ways: by conservative methods or surgically. For decades, closed reduction has been the preferred treatment. However, closed treatment requires varying periods of maxillomandibular fixation (up to 4 weeks) and involves the risk of long-term complications such as pain, ankylosis, internal derangement of the temporomandibular joint, as well as inadequate restoration of the vertical height of the ramus.[34] Therefore, in recent years, there has been an increase in the number of studies indicating that surgery should be the preferred treatment in subcondylar mandibular fractures. The controversy over the treatment of condylar fractures is based on 3 main factors: 1) the age of the frac-

ture incidence; 2) the pattern of deviation of the fracture; and 3) the level of the fracture. In some cases, a conservative approach should still be the preferred treatment.

With this in mind, the current authors employed single applications of 3% and 5% strontium, thinking it would shorten the treatment time in cases undergoing conservative treatment. In the studies mentioned above, local administration of strontium has been effective in orthopedics and dental implants. Similarly, the current study showed that 3% strontium applied over the fracture surface in the mandible accelerated healing histopathologically.

This study is the first to show that local strontium may have positive effects on healing subcondylar mandibular fractures. A local application of 3% strontium chloride hexahydrate has no adverse effect, and accelerates facial skeleton consolidation and bone regeneration. The authors suggest that a combination of strontium chloride hexahydrate and conventional therapeutic approaches may shorten the healing time of subcondylar mandibular fractures in rats.

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Evaluation of NMP22 in bladder cancer patients sensitive to environmental toxins

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Abstract

Background. Bladder cancer (BC) is recognized as environmentally related. The interaction of environmental exposure to chemicals and genetic susceptibility seem to play important roles in BC development. In order to improve diagnosis and the recognition of BC risk, a group of markers which combine genetic susceptibility with detoxification and nuclear matrix protein (NMP22) is proposed.

Objectives. The aim of the study was to examine the utility of nuclear matrix protein (NMP22) as a diagnostic marker in BC in genetic susceptibility (NAT2 slow acetylators) combined with detoxification abilities (glutathione S-transferase GST and isoenzyme GST-π).

Material and methods. The NMP22 level in urine, N-acetyltransferase 2 (NAT2) genotype and GST activity in hemolysate blood, as well as isoenzyme GST-π level, were determined in the urine and serum of 43 patients with BC and from 25 non-cancer controls. NMP22 and isoenzyme GST-π levels were measured by ELISA. The NAT2 genotype was examined in DNA isolated from whole blood using the PCR (Polymerase Chain Reaction) technique, while the activity of GST was determined with the spectrophotometric method.

Results. In the BC group, NMP22 ($p = 0.005$) concentration, GST-π ($p = 0.003$) in urine and GST ($p = 0.009$) activity in blood were statistically significantly higher than in the healthy controls. The majority of BC patients were slow acetylators (NAT2 genotype). A correlation between the level of nuclear matrix protein NMP22 and GST was found in all BC group ($p = 0.007$) and also slow acetylators ($p = 0.0147$).

Conclusions. The results support the utility of a marker combination, which covers the genetic susceptibility to chemicals with the level of detoxification and nuclear matrix protein in BC patients. A relationship between NMP22 level in urine, GST level in blood and NAT2 genotype was observed. Also the isoenzyme GST-π in urine seems useful as a marker of BC.

Key words: bladder cancer, GST, NMP22, NAT2, toxins

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Bladder cancer (BC) is the ninth most common malignancy in the world, the sixth in the USA and the fifth in Europe. According to the Polish National Cancer Registry, it is the fourth cancer among men and the thirteenth among women.[1] There is abundant evidence for the connection between exposure to chemicals and the development of bladder cancer. Since Ludwik Rehn made the groundbreaking observation of increased BC incidence among aniline dye factory workers, many other causative agents have also been identified.[2]

Bladder cancer is responsible for the highest cost of therapy among all the malignancies and, therefore, there is an urgent need for improving its early diagnosis and monitoring. The current diagnostic methods are insufficient, invasive and expensive (cystoscopy, biopsy, urography, computed tomography). Among many proposed potential BC markers, such as bladder tumor antigen (BTA), bladder cancer specific nuclear matrix protein (BLCA), Lewis X antigen, Aurora A kinase, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), ImmunoCyt test, UroVysion, etc., only a few tests have been approved for the routine diagnosis. The Food and Drug Administration (FDA) accepted BTA stat, BTA TRAK, UroVysion and NMP22BladderChek.[3,4]

Our interest has been focused on nuclear matrix proteins as BC in respect of their engagement in chemically induced carcinogenesis. Nuclear Matrix Protein 22 (NMP22) is a non-histone chromatic protein that belongs to the bladder cancer-specific nuclear mitotic proteins. NMP22 is responsible for the proper position of chromatids during mitosis and final separation of daughter cells. It has an important role in ribonucleic acid (RNA) synthesis, deoxyribonucleic acid (DNA) transcription and replication, and in morphological changes of nuclear structure; therefore, it can also have a huge influence on the genes that are crucial in carcinogenesis.[5] The sensitivity of NMP22 as a BC marker is high (the urinary level of NMP22 in patients with BC is up to 25-fold greater than in healthy ones), but the specificity remains insufficient, because false-positive results can be obtained due to urinary infections, urolithiasis and other bladder disorders.[3,4]

Human N-acetyltransferase 2 (NAT2) is a phase 2 drug-metabolizing enzyme that plays an important role in the detoxification of many carcinogens present in the environment.

The NAT2 gene, located on chromosome 8p22, is autosomal dominant. This enzyme exhibits genetic polymorphisms. Different acetylation phenotypes within the population are the result of mutations in the NAT2 gene. Polymorphisms of NAT2 confer slow, intermediate, or fast acetylator phenotypes. The alleles NAT2*5, NAT*6 and NAT*7 are typical for slow acetylators. There are consistent reports on the connection of the NAT2 slow acetylator polymorphisms with higher BC risk, both independently and in association with smoking or occupational exposures, especially to arylamin. There are no reports on the specificity of NMP22 in the slow acetylators group.[6–8]

It seems interesting to explain whether the specificity of the NMP22 marker increases in the BC group of genetically susceptible to environmental toxins slow acetylators with the N-acetyltransferase 2 (NAT2) genotype. The purpose of the study was to select the NAT2 genotype group from BC patients and to examine the NMP22/NAT2 correlation. Many environmental toxins, especially aromatic hydrocarbons, are removed from the body after splitting with glutathione by glutathione S-transferase (GST), so looking for a relationship of BC with environmental exposure, the NMP22/GST correlations were also examined as well as the utility of GST isoenzyme (GST-π) as a marker in BC. These enzymes play an important role in the detoxification of environmental chemicals capable of causing BC.[9,10] GST-π excretion in urine is an important marker of the distal part of renal tubules and loop of Henle damage.[11]

The aim of the study was to examine the utility of the nuclear matrix protein (NMP22) as a diagnostic marker of BC in genetic susceptibility (NAT2 slow acetylators) combined with detoxification abilities (GST and isoenzyme GST-π). Because bladder cancer is described as an environmentally related cancer, it seems necessary to include markers connected with genetic susceptibility and detoxification.

Material and methods

Patients and clinical samples

After their informed consent had been given, first morning urine and blood samples were collected from people who suffered from BC hospitalized in the Department and Clinic of Urology and Urological Oncology of the University Hospital in Wrocław. The BC group consisted of 43 patients (35 men – M and 8 women – W) aged 36–87 years (mean age 69) with histopathologically proved bladder cancer. The BC group was divided into 4 subgroups based on growth – T (stage): Ta (n = 21), T1 (n = 13), T2 (n = 6) and TIS (n = 3). The majority of patients were smokers (67%). About 70.6% among them were urban dwellers and 38.2% reported having had contact with pesticides. Exposure related to the occupation or the place of residence in the industrial area was declared by 41.2% (date from questionnaire). The control group included 25 healthy volunteers (18 men and 7 women) aged 54–81 years (mean age 65) without any urinary tract diseases. The project received the permission of the Bioethics Committee of Wroclaw Medical University (no. KB-292/2013).

Mid-stream morning urine samples were collected in polystyrene containers (Aptaca, Italy) and a urine screening analysis was performed using the dipstick test (Combur 10 Test M, Roche Diagnostics GmbH, Mannheim). Next, the urine samples were centrifuged

for 10 min (1438 × g at 4°C).The obtained supernatant was removed to Eppendorf tubes and stored at –80°C for further investigation.

Blood samples were collected from patients and controls into 2 plastic tubes (BD Vacutainer, USA), one with sodium citrate as an anticoagulant and one without (serum). The tubes were centrifuged at 1438 × g for at least 10 min at 4°C. The supernatant (plasma and serum) was frozen at –80°C until analyzed. The erythrocytes obtained after plasma isolation were hemolyzed in cold phosphate buffered saline and centrifuged at 30065 × g for 15 min at 4°C. The obtained lysate was used for GST measurement.

Immunoenzymatic quantitative analysis (ELISA)

NMP22 concentration in urine

NMP22 concentration was assessed by an enzyme-linked immunosorbent assay (ELISA Kit, Shanghai Sunred Biological Technology Co; CRL; sensitivity 0.25 ng/mL), using NMP22 specific antibodies labeled with biotin, and combined with streptavidin-horseradish peroxidase conjugate to form an immune complex. The absorbance of the samples was then measured with a STAT FAX 2100 spectrometer at λ = 450 nm.[5] The obtained NMP22 level was calculated in relation to the urine creatinine level that had been previously estimated by Jaffe's routine method. Under alkaline conditions, creatinine reacts directly with picric ions to form a reddish complex, the absorbance of which can be measured at λ = 520 nm.

GST-π activity in urine and serum

The concentration of GST-π in urine (ELISA kit, EKF Diagnostics; Ireland; sensitivity 2.0 ng/mL) and GST-π in serum (ELISA kit, Immunodiagnostic AG; Germany; sensitivity 10.7 ng/mL) was assessed by an enzyme-linked immunosorbent assay. This analysis was conducted according to the manufacturer's instructions. The calibration curves were prepared using a purified standard for each protein assessed. Curve fitting was accomplished following the manufacturer's instructions (range 0–40 ng/mL). The absorbance was measured spectrophotometrically using the STAT FAX 2100 spectrophotometer at λ = 450 nm.[12,13] The obtained level of GST-π in urine was then calculated in relation to the urine creatinine level measured previously by Jaffe's routine method.

Polymerase chain reaction (PCR), electrophoresis and acetylation genotype evaluation

The NAT2 polymorphisms and allelic status were determined by the polymerase chain reaction/restriction fragment/length polymorphism.

In order to determine the type of acetylation, the NAT2 genotype was examined in DNA isolated from whole blood using a Blood Mini kit (A&A Biotechnology, Poland). DNA was isolated according to the manufacturer's protocol supplied with the kit reagents. The polymorphic nucleotides at position C481T, A803G, G857A and G590A of the NAT2 coding region were analyzed by digesting the PCR products with the endonucleases *KpnI, Dde, lBamHI, TaqI,* respectively.

The obtained DNA was dissolved in 200 µL of 10 mMTris-HCl buffer (pH = 8.5) and stored in the refrigerator until analysis. Amplification of the NAT2 gene fragment was carried out using a MixPlus 2xPCR kit (A&A Biotechnology, Poland). Primers used in the reaction had the sequence: NAT2 F 5'GCT AGC GGG GGA TCC TCT TC 3 'and NAT2 R 5'TTG GAT GGT TAC ACA ACA AGG G 3'. The amplification primers were used at a concentration of 10 µM each, and the previously obtained DNA was used in an amount of 1 µL. Reactions were carried out according to the program: 94°C – 4 min, 34 cycles: (94°C – 30 s, 59°C – 30 s, 72°C – 45 s), 72°C – 5 min, 8°C – 10 s. The correctness of amplification for NAT2 gene fragments was checked on 2% agarose gel. The restriction digestion of obtained NAT2 gene fragments was carried out in 0.2 mL tubes according to the following scheme: 0.2 µL of enzyme, 0.8 µL of Fast Digest buffer, 2 µL of PCR product and 5 µL of H_2O (Milli-Q system). The reaction was performed for 30 min at 37°C or 60°C for the *Kpnl*, *Ddel*, *BamHI* and *TaqI* enzymes. The wild-type allele (*NAT2*4*) was identified after complete digestion by *Kpn*1, *Taq*1, *Bam*H1 and *Ddel*. Genetic variation in the *NAT2* gene changed the recognition sites for *Kpn*1, *Taq*1, *Bam*H1 and *Ddel* restriction enzymes. *NAT2*5*, *NAT2*6,* and *NAT2*7* alleles were identified by the presence of C481T, G590A, G857A and A803G genetic variations, respectively.

According to the obtained results, the BC patients were divided into three categories: I – with slow acetylation genotype SA (*5/*5,*5/*6,*5/*7,*6/*6.*6/*7); II – intermediate acetylation genotype IA (*4/*5,*4/*6); III – fast acetylation genotype FA (*4/*4).

Spectrophotometric method of enzymatic activity evaluation

The activity of GST was determined with the spectrophotometric method (GST Assay Kit, Sigma, USA) based on the conjugation of the thiol group of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB). The absorbance of the colored product was measured with a STAT FAX 2100 spectrophotometer at λ = 340 nm.[15] The hemoglobin level in hemolysates was also evaluated using routine Drabkin's method and then the GST level was calculated in relation to the amount of hemoglobin in the sample.

Statistical analysis

Statistical analysis was conducted with Statistica PL software (v. 10.0). The variability of distribution was checked with the Lilliefors and the Kołomogorov-Smirnov tests. For variables with normal and non-normal distribution, Student's t-test and U Mann-Whitney test were used, respectively. Correlations were assessed by the Pearson/Spearman correlation analysis. A p-value of less than 0.05 was considered statistically significant.

Results

Nuclear matrix protein 22

There was a statistical difference, an almost 2 times higher level of NMP22, expressed as ng/mg creatinine, in patients with bladder cancer than in the control group (p = 0.005) (Fig. 1). The concentration of NMP22 was up to 20% higher in the group of smokers compared to non-smokers. Women had more than 1.5 times higher concentration of NMP22 compared to men, but this difference was not statistically significant (Fig. 1). No correlation between the level of NMP22, BMI, erythrocyturia and proteinuria was found. The analysis of the association between NMP22 concentration and cancer stage was not completed because of the small size of subgroups (e.g. 21 persons with Ta, 13 with T1, 6 with T2, 3 with TIS); however, the mean urinary level of NMP22 was the highest in T1 stage (14.34 ng/mg cr.) and TIS (14.82 ng/mg cr.) and it was almost 2 times lower in Ta (9.89 ng/mg cr.) (Fig. 1).

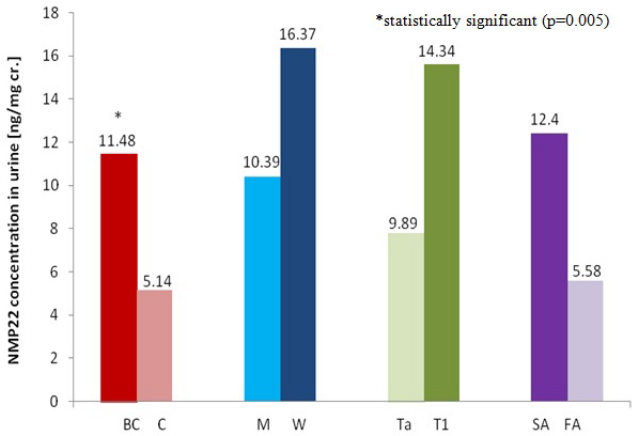
N-acetyltransferase 2 genotype (NAT2)

The analysis of the NAT2 genotype in BC patients revealed the presence of 4 mutations (Table 1).

1. C481T mutation revealed the presence of a wild type genotype (wt) in 1 patient, the genotype with 1 mutated allele (ht) in 33 patients, and 2 mutated alleles (mt) in 9 patients. The frequency of the mutant C481T allele of the patients was 97.7%
2. A803G mutation corresponding values were respectively wt – 15; ht – 21; mt – 7. The frequency of the mutant A803G allele of the patients was 65.1%
3. G857A mutation corresponding values were respectively wt – 34; ht – 9; mt – 0. The frequency of the mutant G857A allele of the patients was 20.9%
4. G590A mutation corresponding values were respectively wt – 16; ht – 24; mt – 3. The frequency of the mutant G590A allele G590A of the patients was 62.8%.

There were 8 different genotypes of NAT2 in the examined group of BC patients. Three people (6.9%) with NAT2 *4/*4 genotype were classified as fast acetylators (FA), 12 patients (27.9%) with NAT2 *4/*5 and *4/*6 genotype as intermediate acetylators (IA), and 28 patients (65%) with NAT2 *5/*5, *5/*6, *5/*7, *6/*6 and *6/*7 as slow acetylators (SA). The obtained results indicated that the slow acetylation genotype dominated in the BC patient group (Table 2).

Fig. 1. NMP22 concentration in urine of bladder cancer patients (BC) and controls (C)



M – men; W – women; Ta, T1; SA – slow acetylators; FA – fast acetylators.

The correlation between NMP22 concentration and acetylation genotype was examined. No significant correlation was found, although the mean NMP22 urinary levels in each group – fast (FA), intermediate (IA) and slow (SA) acetylators – were different, the highest in slow acetylators (12.44 ng/mg cr.) and the lowest in fast acetylators (5.58 ng/mg cr.) (Table 3, Fig. 1).

No statistically significant correlation was found between the acetylation genotype and cancer stage. However, in the group of slow acetylators the predominant stages were Ta (42.9%) and T1 (36%).

Glutathione S- transferase and isoenzyme π

The analyses of glutathione S-transferase (GST) activity and its isoenzyme π in the blood of BC patients and the control group were shown (Table 1). There was a statistically significant increase of GST activity in the BC group compared with the control group (3.20 and 2.39 µmol/g Hb, respectively) (p = 0.009). The analyses of the correlation

Table 1. The analysis of four NAT2 SNP tested of BC patients

NAT2 polymorphisms	C481T *KpnI* n (%)	A803G *DdeI* n (%)	G857A *BamHI* n (%)	G590A *TaqI* n (%)
wt	1 (2.3)	15 (34.9)	34 (79.1)	16 (37.2)
ht	33 (76.7)	21 (48.3)	9 (2.9)	24 (55.8)
mt	9 (2.9)	7 (16.3)	0 (0)	3 (1.29)

wt – wild type; ht – heterozygotus mutant; mt – homozygotus mutant; SNP – single nucleotide polymorphisms; *KpnI, Dde,I BamHI, TaqI* – restriction enzymes.

Table 2. The analysis of genotype and acetylation status in BC patients

Genotype	Acetylation status	Number: n (%)
*4/*4	fast	3 (6.9)
*4/*5	intermediate	4 (9.3)
*4/*6	intermediate	8 (18.6)
*5/*5	slow	6 (13.6)
*5/*6	slow	12 (27.9)
*5/*7	slow	3 (6.9)
*6/*6	slow	3 (6.9)
*6/*7	slow	4 (9.3)

between the urinary level of NMP22 and the blood level of GST in BC patients showed a statistically significant positive correlation (r = 0.41 p = 0.007) (Fig. 2).

Also, a significant difference in the isoenzyme GST-π activity, but only in urine, not in the serum, was noted for BC patients in comparison to the control group (C) (p = 0.003). The mean level of GST-π in urine in the BC and C group was 28.98 and 4.6 ng/mg creatinine, respectively (Table 3). A positive NMP22/GST-π urine level correlation was noted only for T1 stage (13 cases), not for the whole BC group (r = 0.818; p = 0.001).

The correlation between NAT2 genotype and the activity of GST and GST-π in BC patients was also assessed (Table 3). The level of GST was lower in the SA group (28 patients) than in IA (12 patients) and FA (3 patients) acetylators (2.98; 3.54 and 3.98 ng/g Hb respectively) (Table 3), but the differences were not statistically significant, including in relation to the activity of isoenzyme GST-π (Table 3).

With regard to NAT2 genotype, a NMP22/GST correlation was found only in the slow acetylator group (r = 0.46, p = 0.0147). No significant NMP22/GST-π correlation in SA, IA or in FA acetylators was found.

Fig. 2. The positive correlation between GST and NMP22

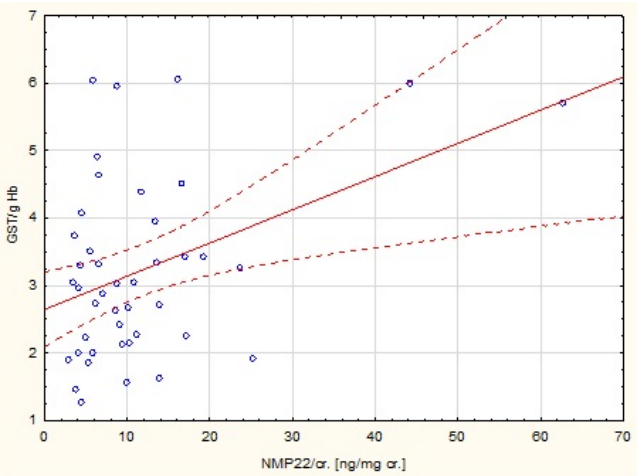


Table 3. The values of NMP22, GST, GST- π in subgroups depending on the acetylation genotypes

Acetylation genotype/ values in BC and C	NMP22 [ng/mg crt.] (urine)	GST [μmol/g Hb] (blood)	GST-π [ng/mL] (serum)	GST- π [ng/mg crt.] (urine)
SA				
$\bar{x}$	12.44	2.96	216.82	29.72
SD	11.61	1.09	159.51	38.92
IA				
$\bar{x}$	11.22	3.54	214.43	35.13
SD	10.91	1.53	99.89	32.36
FA				
$\bar{x}$	5.58	3.98	205.30	12.85
SD	1.07	2.45	72.97	12.73
BC				
$\bar{x}$	11.48*	3.20*	225.37	28.98*
SD	10.95	1.32	142.00	35.17
C				
$\bar{x}$	5.15	2.39	192.08	4.62
SD	2.03	0.84	68.07	3.43

* statistically significant activity in comparison to control group; SA – slow acetylation; IA – intermediate acetylation; FA – fast acetylation in BC (bladder cancer patients group); C – control group.

Discussion

Cystoscopy with the cytology of urine has been a standard procedure in the detection of bladder cancer. It is also used in the current surveillance protocol every 3 months for 1–3 years of therapy, every 6 months for the next 1–3 years, and then annually thereafter. This is costly and invasive to the patient. The detection of BC is still an important global medical problem. In the last years, several new urine markers have been developed for urothelial carcinoma detection. They should be non-invasive, have high sensitivity, high specificity and a positive predictive value to avoid unnecessary cystoscopies. NMP22 was considered as a promising marker, accepted by the FDA, but critical opinions are also published nowadays.[16] The NMP22 test has been evaluated as a non-invasive (urine), qualitative (ELISA) and quantitative (Bladder Check) method; the latter in particular is accepted for disease monitoring. It was observed that a bladder cancer patient has a higher amount of NMP22 in urine. However, the specificity of the marker NMP22 was low, because this protein is released from dead urothelial cells and could give false positive results, e.g. in an inflammatory state.[17,18] The review of bladder cancer markers led to the conclusion that there is no single one that could be recommended for reducing cystoscopy frequency. It seems that the best way to improve BC detection is to look for a combination of tumor markers. Bladder cancer is an environmentally related cancer. Many chemicals are able to induce BC.[2] Tobacco smoking is a major cause of BC in humans.[19] The interaction of environmen-

tal exposure and genetic susceptibility seems to be an important cause of BC. An increased risk of BC was observed for smokers with NAT2 slow acetylation genotype.[20,21] The genetic polymorphisms of enzymes involved in toxication (activation) or the detoxification of environmental toxins play a significant role in the individual susceptibility to BC, particular in regards to smokers. There are some reports on the association of NAT2 slow acetylator genotype and the risk of BC in the exposure to aromatic amines, but there are also reports where the association was not found.[22,23] The slow acetylator status was proved as a genetic risk factor for arylamine-induced bladder cancer for the European (Caucasian) population, but not for the Chinese population.[24]

In our study a significantly higher (over twofold) urinary level of NMP22 was noted in patients with BC compared to the control group. The majority of patients (61%) were slow acetylators. Although we did not find a significant correlation between NMP22 level and NAT2 genotype, an upward trend was observed: from the lowest NMP22 concentration in fast acetylators to the highest NMP22 concentration in the urine of slow acetylators. The study was limited by a small numbers in the subgroups. It was also found that the frequency of NAT2 slow acetylator genotype was significantly higher in BC patients than in the normal group.[25] Also, in our group of BC patients, the slow acetylator genotype was predominant.

The relationship between environmental exposure to several chemicals and bladder cancer development is known. Tobacco smoking is the cause of almost half of all BC cases and is connected with the exposure to numerous carcinogens in tobacco smoke, such as hydrocarbons, arylamines, formaldehyde, acrylonitrile, cancerogenic metals and free radicals.[1,26] Many aromatic amines can cause bladder cancer. It is also known that slow acetylators are more sensitive to aromatic amines and other agents present in the environment.[2] Examination of NAT2 mutation deserves special attention, because of its role in detoxification by N-acetyltransferase activation, especially through the NAT2 regulated pathway.[27] It is known that the NAT2 slow genotype is characterized by the decreased acetylation of aromatic amines, which increases the risk of bladder cancer, especially in heavy smokers.[28]

The aim of our study was to examine whether the combination of markers which join the genetic susceptibility to chemicals with the level of detoxification and NMP22 is useful in BC diagnosis and whether the specificity of NMP22 is high enough in BC patients with the slow acetylation genotype NAT2 and could be helpful in early diagnosis of BC. To our knowledge no one has examined the specificity of NMP22 in regards to the group with a genetic susceptibility to environmental toxins. It seemed interesting to assess the relation between genetic susceptibility and the activity of enzymes which play an important role in detoxification (GST, GST-π) and urinary NMP22 quantity. There was a preliminary study evaluating the utility of NMP22 for prophylactic screening of early recurrence of disease for patients with genetic susceptibility. There is some evidence of the greater importance of NMP22 estimation in risk groups, e.g. smokers and the elderly. Investigations of Cui et al. indicate that the NAT2 slow acetylator genotype can decrease the detoxification of tobacco mutagens, such as arylamines, and be synergistic with other metabolizing enzymes.[29] The authors suggest that individual genetic variations affecting the metabolic activity of enzymes involved in arylamine detoxification could modify the risk of bladder cancer. Also, the role of nicotine-induced cell proliferation is reported.[30]

Glutathione S-transferase is involved in the biotransformation of many substances qualified as risk factors for BC, specially aromatic and halogenated hydrocarbons. GST catalyzes the conjugation of electrophilic toxins with glutathione. Some studies have reported that the GSTT1 null genotype was associated with an increased risk for BC.[31] Thus, changes in the expression and activity of GST influence the individual resistance or susceptibility to xenobiotic-induced damage.[32] According to Saygili et al., the stimulation of GST expression in people with BC is a response to DNA damage caused by various environmental factors.[33] The results of our study showed significantly higher total GST activity in the BC group in comparison to healthy volunteers. Although no statistically significant differences were found for the isoenzyme, GST-π in serum and a significant difference in the GST-π level in urine suggest that isoenzyme π is also involved in the examined processes. The investigations have revealed increased detoxification among BC patients and suggest that GST could be an additional environmental related marker in BC. The statistically positive correlation between NMP22 and GST points to a relationship between the intensity of detoxification and nuclear matrix protein level. The examination of NMP22/GST correlation in the 3 acetylator groups SA, IA and FA gave a statistically significant result only in the SA group ($r = 0.4$, $p = 0.015$). This suggests higher specificity of the marker NMP22 for slow acetylators, i.e. BC people susceptible to environmental toxins. This research should be continued. Simic et al. indicate that the upregulation of various GST classes, especially π and θ isoforms, might have important consequences for transitional cell carcinoma (TCC) of bladder growth by providing a reduced cellular environment and the inhibition of apoptotic pathways (by inhibition of JNK kinase).[34] Moreover, the occurrence of low GST-π type of conjugation can be considered as a risk factor of TCC. For people with lowered GST-π activity, exposure to arsenic and PAH is more dangerous than for others.[35] The role of hereditary polymorphisms of NAT and GST genes involved in the etiology of neoplasm of the urinary tract is still controversial. Some authors state that both NAT and GST polymorphisms are

responsible for the different ability to metabolize carcinogens, especially those in tobacco smoke.[36] The correlation between total GST activity in serum and NMP22 concentration in urine suggests that these parameters may be useful for assessing the exposure to carcinogens among BC patients susceptible to environmental toxins and probably for evaluating the predisposition to BC development. Thus, further research including a larger number of people should be carried out.

Further prospective research is needed to confirm these relationships and to prove the usefulness of these parameters in the prognosis of BC development and/or evaluation of BC morbidity, which is crucial in the aspect of race NAT2 polymorphism and the susceptibility to BC.

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Correlations between iron content in knee joint tissues and chosen indices of peripheral blood morphology

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Abstract

Background. Iron as a cofactor of enzymes takes part in the synthesis of the bone matrix. Severe deficiency of iron reduces the strength and mineral density of bones, whereas its excess may increase oxidative stress. In this context, it is essential to determine the iron content in knee joint tissues.

Objectives. The study objective was to determine the level of iron in the tissues of the knee joint, i.e., in the femoral bone, tibia and meniscus.

Material and methods. Material for analysis was obtained during endoprosthetic surgery of the knee joint. Within the knee joint, the tibia, femur and meniscus were analyzed. Samples were collected from 50 patients, including 36 women and 14 men. The determination of iron content was performed with the ICP-AES method, using Varian 710-ES.

Results. The lowest iron content was in the tibia (27.04 µg/g), then in the meniscus (38.68 µg/g) and the highest in the femur (41.93 µg/g). Statistically significant differences were noted in the content of iron in knee joint tissues.

Conclusions. In patients who underwent endoprosthesoplasty of the knee joint, statistically significant differences were found in the levels of iron in various components of the knee joint. The highest iron content was found in the femoral bone of the knee joint and then in the meniscus, the lowest in the tibia. The differences in iron content in the knee joint between women and men were not statistically significant.

Key words: iron, bone tissue, tibia, femur

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Iron is a cofactor in many enzymes and cell redox reactions. A low level of iron ions may be harmful for the cell, whereas an excessive amount may cause the generation of reactive oxygen species via Fenton's reaction. Cellular iron content is strictly regulated by homeostatic mechanisms to maintain its adequate level in the cell. The ions of nickel and other divalent metals can compete with iron ions to enter the cell through divalent metal transporter 1 (DMT1), as they have similar ion rays. Therefore, metal ions can affect many other cell iron-dependent processes.[1]

Iron as an enzymatic cofactor takes part in the synthesis of the bone matrix (lysyl hydroxylase activation) and 25-hydroxycholecalciferol hydrolase.[2] Moreover, Fe ions aided by active vitamin D stimulate the absorption of Ca ions in the intestine. Iron deficiencies in rats led to poor mineralization of their skeletons and pathological lesions in the microarchitecture of the spongy matter of the vertebrae.[3,4] In turn, estrogen administration increased the accumulation of iron in hamsters and facilitated its intake by lymphocytes in culture.[1,5]

Deficiency of Fe ions in young rats led to a decrease in mechanical strength of the femoral bone and cortical bone.[6] In severe deficiency, strength and mineral density of the bone was reduced.[7]

An excess of iron content in mice led to increased oxidative stress, which mediates bone mass loss through bone rebuilding.[8]

In rats with severe anemia due to iron deficiency, the concentration of procollagen type I N-terminal pro-peptide was low, which caused a decrease in bone matrix formation and mineralization.[9] These parameters returned to normal following the intake of dietary iron.[10] No data is available on the significance of iron for the health of bones in humans. On the other hand, osteopenia was observed in patients with genetically conditioned hemochromatosis and a very high iron content in tissues.[11] Thus, the protective or destructive effects of iron on bones depend on its concentration.[2]

Anemia due to iron deficiency has a great impact for health. Deficiency of iron ions in women at reproductive age and in adolescents may also affect bone health at the time when peak bone mass is acquired.[12]

Results reported by Harris et al. indicate that an increase in the level of iron can be essential to prevent fractures, especially in some groups, such as elderly women, former female athletes and female army workers.[13]

As shown by a recent study, patients with osteoporosis have iron deficiency, i.e., a lower concentration of iron ions and higher serum transferrin level, as compared to a control group.[14]

Additionally, rats with general iron deficiency have lower bone mass than animals with high iron level. However, no such correlation has been noted in people.

An increasing number of studies suggest the existence of correlations between lipid oxidation and bone metabolism, as well as between iron metabolism and LDL oxidation. The availability of iron ions for cells also depends on the haptoglobin phenotype.[15] It has been found that in postmenopausal women, Hp 1.1 and 2.2 are associated with greater risk of fractures as compared to Hp 2.1. The relationship between Hp and bone mass results from its role in iron metabolism and antioxidant properties, since serum iron levels in patients suffering from osteoporosis are lower than in healthy control subjects. The patients had greater amounts of oxLDL (oxidized low-density lipoproteins) as compared to the controls.[15]

Zheng et al. revealed that low serum levels of iron were associated with osteoporosis.[8] Previous studies have reported that patients with osteoporosis suffer from iron deficiency, which in turn may decrease iron bioavailability and affect bone metabolism. In that case, iron acts as a cofactor in the enzymes involved in the synthesis of intracellular substances of the bone, and in the vitamin D enzyme and hydroxylase, engaged in vitamin D activation. Animal studies indicate that Fe deficiency is accompanied by a reduced level of osteocalcin, the mineral content of bones, lowered density of bone tissue and mechanical strength of the femur. Iron loss in the culture of osteoblasts induced mineralization disorders, similar to those observed in some human populations. Mineralization disorders induced by iron deficiency seem to be a possible mechanism in osteoporosis.[8]

The above literature survey shows a major role of iron content in the human body. However, no information is available on the role of iron ions in the tissues of the knee joints in relation to chosen morphological indices as the main pathway of general iron distribution.

Material and methods

The study material included parts of the knee joint obtained during endoprosthesoplasty in the Dr Janusz Daab District Hospital of Orthopedics and Trauma Surgery in Piekary Śląskie, Poland. Biological samples were obtained from patients living in Silesia Province. Samples were collected from 50 patients, 36 women and 14 men. In 26 patients the right leg and in 24 patients the left leg was involved. The mean age of the whole study population was 67.5 years, being slightly lower in women (67.2 years), than in men (68.1 years).

Degenerative disease of the knee joint and considerable pain were indications for this type of procedure. In the study group, the patients complained of pain of 10 years' duration on average. The study was approved by the Bioethics Committee, no. 2/2013 of June 18th, 2013.

Surgeries were performed under subarachnoid anesthesia, with patients in the prone position. Esmarch bandage was used for exsanguination of the limb. The frontal surface of the knee joint was exposed, following the standard preparation of the operation field (applying antiseptic and aseptic techniques) with straight midline incision. The joint was opened at the medial side and the hypertro-

phic synovium was removed. Using ZIMMER instrumentation, the femoral part of the knee joint was prepared by processing the distal femur and performing femoral epicondyle osteotomy. Next, damaged menisci were removed and the tibial part was prepared using ZIMMER instrumentation (resection of the tibial plateau). In this way, the osseous components, cartilages and parts of menisci were used for measurements.

The material samples were described and stored in modified polyethylene containers, in a freezer at a temperature of -22°C.

Tissue samples with a known mass were mineralized using 4 cm^3 of spectrally pure HNO_3 (V) (Supra pure, Merck, Dormstadt, Germany), in a Magnum II (ERTEC) microwave mineralizer. The samples were placed one by one in a Teflon vessel and submitted mineralization. Mineralization was a 2-stage procedure. The 1st stage lasted 2 min at 20 bar max pressure and 255°C max. temperature, whereas the 2nd stage was of 6 min duration at 45 bar max pressure and 285°C max. temperature. The post-mineralization solution was transferred to a 25 cm^3 flask and then diluted to the mL mark with redistilled water.

The content of iron in the mineralized samples was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES). A Varian 710-ES spectrometer equipped with a OneNeb nebulizer was utilized. The following parameters were used: RF power 1.0 kW, plasma flow 15 L/min, auxiliary flow 1.5 L/min, nebulizer pressure 210 kPa, pump rate 15 rpm, emission lines of Fe: λ = 238.204 and 259.940 nm. The calibration curve method was applied. The standard solutions of 1 mg/mL (Millipore SAS, Molsheim, France) as well as deionized water (Elix Essential 10) were used. The results are an average of the concentrations obtained for all analytical lines used for the element, with standard deviation not exceeding 1.5%. The accuracy of the analysis was controlled using Standard Reference Material 1400 Bone Ash (NIST).

Hematological indices were marked on the BC-5380 hematology analyzer, number RF-21101836, year of production: 2012. The analyzer is under constant supervision of an authorized service, and undergoes annual inspection and service-maintenance procedures. Quality control is conducted internally (daily at 3 levels: low, normal and high, 365 days a year) and externally (24 times a year) (RIQAS).

The study objective was to determine the level of iron in the tissues of the knee joint, i.e., in the femoral bone, tibia and meniscus. Statistical analysis of the results was performed separately for women and men, with a division into the right and left limb. Correlation analysis was used to assess the role of iron content in relation to chosen morphological parameters of the peripheral blood measured on the day of admission to hospital. The aim of the research was also to determine factors that may affect the content of iron in tissues of the knee joint, i.e., smoking and previously implanted prostheses.

Statistical analysis

The analysis used STATISTICA PL v. 10.0 (StatSoft, Inc., Tulsa, USA) software.

To determine the distribution of the results, the Shapiro-Wilk's test was used ($p < 0.05$). The distribution of results was an abnormal distribution.

The results of the study were presented in arithmetic mean values. The tables that describe the content of iron in the tissues of the knee contained: arithmetic mean, standard deviation, median, and range of changes. Hematological indices were presented by the arithmetic mean, standard deviation, median, range of changes, and the coefficient of variation.

The analysis of differences in iron concentration between different treatment groups was made using the Mann-Whitney U-test.

To evaluate differences in the content of iron in various tissues of the knee joint, the Kruskal-Wallis ANOVA rank test was used. The same test was used in analyzing the differences in the iron content in the population of smokers and non-smokers, and in different age groups.

Additionally, Spearman's rank correlation between iron and the hematological indices in the population of women and men was determined. Significant rho correlation coefficients occurred at a probability level of $p < 0.05$.

Results

The results related to iron content in the chosen tissues of the knee joint have been presented in Fig. 1. The lowest iron content was in the tibia (27.04 μg/g), then in the meniscus (38.68 μg/g), and the highest in the femur (41.93 μg/g) (Table 1). Statistically significant differences were noted in the content of iron in knee joint tissues (ANOVA Kruskal-Wallis rank test, $p = 0.052$).

No statistically significant differences were noted in iron content in the tibia between women and men. The Fe levels were higher in women than in men. In the femoral bone, the levels of iron were also higher in women than in men. In the meniscus, higher iron content was found in men than in women, although the differences were not statistically significant (Table 2).

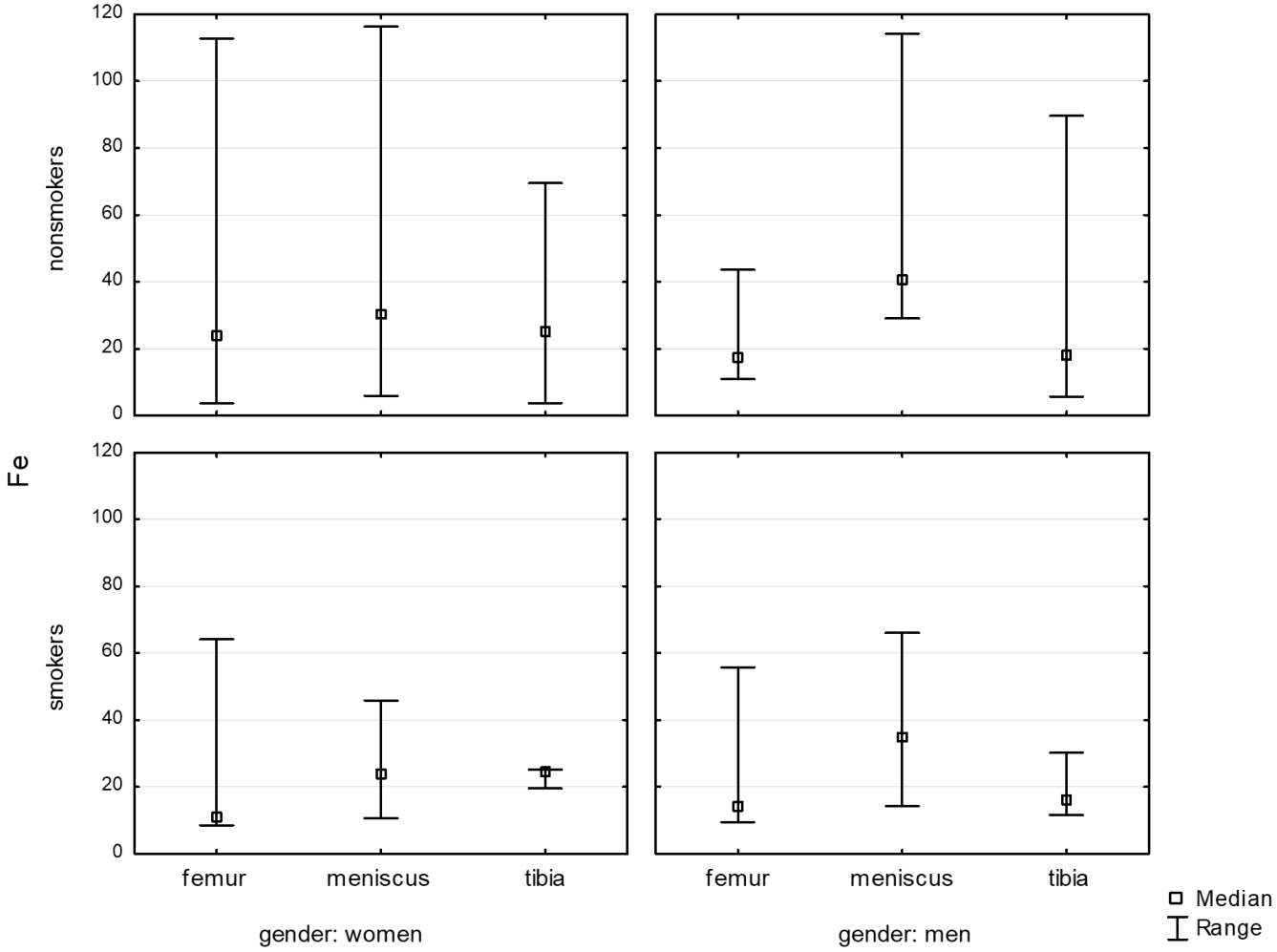
In both women and men, no statistically significant differences were observed between the tissues of the left and right limb (Table 3).

Table 1. Iron content in knee joint tissues (μg/g)

	AM ±SD	Med	Range
Tibia n = 50	27.04 ±22.04	20.07	2.32–99.27
Femur n = 50	41.93 ±67.44	17.20	3.69–410.01
Meniscus n = 50	38.68 ±29.66	29.61	5.90–118.82

AM – arithmetic mean; SD – standard deviation; Med – median.

Fig. 1. Iron content in knee joint tissues in female and male smokers and nonsmokers



The comparison of the averages for both genders combined showed that the Fe content was higher in nonsmokers (39.11 μg/g) as compared to smokers (25.47 μg/g). In the group of female smokers, the content was close to that observed in male smokers (24.43 μg/g vs 26.50 μg/g). However, in the group of non-smoking women, the level was 39.68 μg/g vs 36.40 μg/g in men (Fig. 1).

In patients who had already undergone endoprosthesoplasty of the other knee, the content of Fe was 32.81 μg/g, which was lower than in patients who had never had that type of surgery (36.96 μg/g). However, the differences were not statistically significant.

There were statistically significant differences in the content of iron in the tissues of the knee joint according to age (Kruskal-Wallis ANOVA Rank Test, p = 0.055).

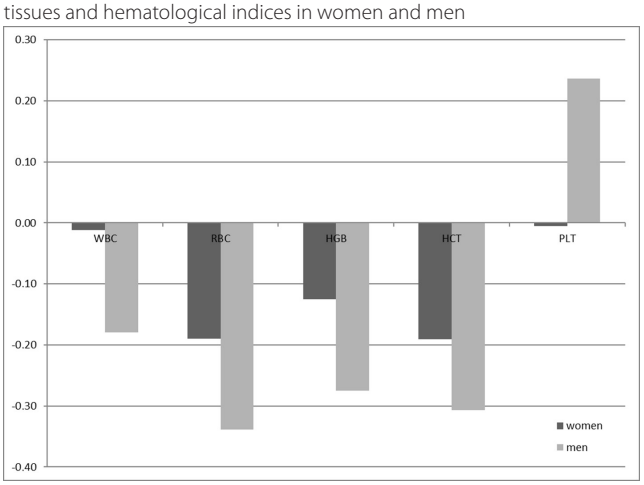
The chosen morphological parameters have been presented in Table 4. In women, the values of all hematological indices were lower as compared to men.

Table 2. Iron content in knee joint tissues in women and men (μg/g)

		Tibia n = 36	Femur n = 36	Meniscus n = 36
Women	AM ±SD	29.05 ±22.30	43.42 ±73.80	36.61 ±27.54
	med	23.13	17.45	28.5
	range	2.32–99.27	3.69–410.01	5.90–116.34
Men	AM ±SD	21.88 ±21.26	38.08 ±49.57	44.00 ±35.08
	med	16.3	16.45	37.45
	range	5.71–89.62	9.45–197.22	7.48–118.82
Women vs men	M-W test	ns	ns	ns

AM – arithmetic mean; SD – standard deviation; med – median; M-W test – Mann-Whitney U test; ns – non-significant difference.

Fig. 2. Spearman's correlation analysis between iron content in knee joint tissues and hematological indices in women and men



The correlation analysis showed a relationship of the level of Fe in the knee joint with RBC in women (rho = -0.19) and men (rho = -0.34) and with HCT in women (rho = -0.19) and men (rho = -0.31) (Fig. 2).

Discussion

Articular degeneration is a chronic, non-inflammatory disease of multifactorial etiology. As a result of chronic joint destruction, the patient suffers from locomotor dysfunction and complains of impaired performance and difficulty in everyday functioning. As a consequence, the quality of life is reduced.[16]

Bone tissue, due to long restoration and retention time, is considered a biomarker of exposure to trace elements.[17–20] Bones are characterized by a very slow exchange of elements, whose biological half-lives are estimated to be a few years to several decades. The content of elements in bone tissue and of some trace elements have been frequently assessed in numerous studies.[18–23]

The comparison of gender-related iron concentrations showed that the lower level of the element in the knee joint of women as compared to men was in the meniscus. The iron content in the femur and tibia was found to be higher than in men. In the knee joint tissues, a lower iron concentration in women was observed only in the articular cartilage, whereas it was higher in all other components of the joint in women. The reasons for the statistically significant differences in the iron content in the tissues of the knee joint are the diverse metal accumulation capacities of each type of tissues, the location of these tissues and environmental exposure.

Iron concentrations in both populations were very similar. No statistically significant differences were found in the femur, tibia and meniscus between patients without knee endoprosthesis and those with implanted endoprosthesis. A comparison of the respective tissues in the knee joint showed a higher content of iron in the meniscus in patients with knee endoprosthesis (44.50 µg/g) than in those without one (36.63 µg/g). In the femur and tibia, the level of iron was higher in subjects without endoprosthesis (33.15 and 27.59 µg/g; 28.44 and 25.49 µg/g, respectively).

In nonsmokers, the levels of iron in the femur, tibia and meniscus differed statistically significantly (ANOVA Kruskal-Wallis rank test, p = 0.088). The differences were not observed in smokers.

The reason for differences in the content of iron in the tested tissues is likely to result from ongoing degenerative changes in the knee joint. Consequently, there are abnormalities in the blood supply of tissues, which may have an impact on the content of elements in these tissues.

It turned out that the content of iron determined in the knee joint of patients living in Silesia Province was

Table 3. Iron content in the right and left knee joint in women and men (µg/g)

		Women			Men		
		tibia n = 18	femur n = 18	meniscus n = 18	tibia n = 8	femur n = 8	meniscus n = 8
Right limb	AM ±SD	24.76 ±18.86	37.65 ±50.06	36.49 ±23.13	15.97 ±9.58	26.67 ±16.81	42.00 ±36.69
	med	17.19	16.53	28.5	11.67	19.61	32.92
	range	2.32–66.77	4.76–205.48	12.44–90.87	6.08–30.97	10.49–55.77	9.48–118.82
		tibia n = 18	femur n = 18	meniscus n = 18	tibia n = 6	femur n = 6	meniscus n = 6
Left limb	AM ±SD	33.33 ±25.08	49.20 ±92.93	36.73 ±32.04	29.77 ±30.27	53.29 ±74.21	46.65 ±36.07
	med	24.6	17.97	30.1	19.91	15.84	39.95
	range	3.79–99.27	3.69–410.01	5.90–116.34	5.71–89.62	9.45–197.22	7.48–114.19
	M-W Test	ns	ns	ns	ns	ns	ns

AM – arithmetic mean; SD – standard deviation; med – median; M-W test – Mann-Whitney U test; ns – non-significant difference.

Table 4. Chosen morphological parameters of the peripheral blood in women and men undergoing endoprosthesoplasty of the knee joint

		AM ±SD	Med	Range	CV
Women	WBC	6.87 ±1.61	6.64	4.14–10.83	23
	RBC	4.54 ±0.47	4.61	3.30–5.34	10
	HGB	13.37 ±2.02	13.80	4.54–15.70	15
	PLT	238.89 ±63.36	235.00	103.00–341.00	27
	HCT	41.04 ±3.83	41.75	32.50–47.90	9
Men	WBC	7.51 ±2.35	7.42	4.45–13.40	31
	RBC	4.79 ±0.37	4.94	4.22–5.24	8
	HGB	14.52 ±1.02	14.50	12.40–16.20	7
	PLT	254.21 ±72.63	263.00	155.00–400.00	29
	HCT	43.31 ±3.05	43.75	36.30–47.90	7

AM – arithmetic mean; SD – standard deviation; Med – median; CV – coefficient of variation.

higher as compared to the results reported by Kuo et al., relating to the bones of inhabitants of Taiwan – 20.3 μg/g.[24]

For comparison, the iron content in the bones determined by Bush et al. was 54 μg/g on average, slightly higher than in our study.[25]

In other bone tissues, e.g., the ribs, iron content was 140 mg/kg, a few times higher than in our study.[26] Subsequent findings reported by Scancar et al. confirm the correlation between iron content and the type of bone tissue, e.g., in the iliac crest it was 100–300 mg/kg and, as shown by Takata et al., in the cancellous bone of the ribs it was 377 μg/g.[27–28]

Iron concentration in the knee joint is a few times lower than in the hip joint. The discrepancy is due to the application of different methods: ICP-AES in the knee joint and AAS in the hip joint. Moreover, in the latter case, iron level was determined in the head of the femur with division into articular cartilage, cortical bone and spongy bone. In the knee joint, measurements were done in the femur and tibia, with no division into tissues. For instance, in the articular cartilage of the femoral bone, iron concentration was 183.75 μg/g, in the cortical bone 111.16 μg/g, and in the spongy bone 163.25 μg/g.[29] However, the content of iron was 31.93 μg/g in the femur, 27.04 μg/g in the tibia, and 38.68 μg/g in the meniscus.

Iron content in bone tissues can also vary geographically, which has been confirmed by the results reported by Yoshinaga et al. on iron content in rib bones (71.0 μg/g).[30] Like in our study, they found no statistically significant differences between women and men.

Assessment of the content of elements in the bones obtained from archeological excavations is commonly reported. Vuorinen et al. determined that Fe content in the ribs of skeletons found in excavations was 5691 μg/g on average.[31] The iron level was lower in the group of women (5564 μg/g) as compared to men (6914 μg/g).

Research into the content of iron conducted by Helliwell et al. in the femoral bone in people with fractures and degenerative lesions is methodologically comparable with the current results.[32] A considerably higher content of iron was noted in patients with fractures (275.2 μg/g) as compared to degenerations (115 μg/g).

The data reported by Kosuga et al. refers to the content of elements in the bones (ribs) from Japan dated back to 120–5000 years B.C.[33] The level of Fe was found to undergo changes from 104 μg/g to 10970 μg/g, being the lowest in the Kofun epoch and the highest in the Edo epoch.

In a study performed by Hisanaga et al., Fe content in excavated bones ranged from 35.5 μg/g to 2793.8 μg/g, being the highest in the Edo epoch.[34]

In this part of the discussion, it should be emphasized that the iron content in bones from excavations is many times higher as compared to the levels observed in our study. The content of iron was also affected by diagenesis processes taking place in the soil, as well as the use of iron-made vessels and goods.

Conclusions

In patients who underwent endoprosthesoplasty of the knee joint, statistically significant differences were found in the levels of iron in various components of the knee joint.

The differences in iron content in the knee joint between women and men were not statistically significant. The highest iron content was found in the femur of the knee joint, then in the meniscus, and the lowest in the tibia. However, the differences were not statistically significant.

Smokers had lower Fe content in the knee joint tissues than nonsmokers.

A correlation was found between iron content in the knee joint and RBC in women (rho = -0.19) and men (rho = -0.34), and HCT in women (rho = -0.19) and men (rho = -0.31).

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Tumor marker α-fetoprotein receptor does not discriminate between benign prostatic disease and prostate cancer

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Conflict of interest
RECAF concentration in patients' blood samples was measured free of charge by BioCurex, Inc., Suite 215, 7080 River Road, V6X 1X5 Richmond, BC, Canada. Biocurex Inc. had no role in the design of the study, the collection and analysis of the data or the decision to publish. No other conflicts of interest are declared.

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Abstract

Background. The α-fetoprotein receptor (RECAF) is a proposed novel tumor marker for detecting several different types of tumors, including prostate cancer (PCa).

Objectives. The aim of the study was to evaluate RECAF in discriminating benign prostatic conditions from PCa and to compare it with prostate-specific antigen (PSA).

Material and methods. A total of 64 patients with elevated serum PSA levels and/or abnormal digital rectal examination of the prostate referred to a tertiary center for transrectal ultrasound (TRUS) biopsy of the prostate were prospectively enrolled in the study from January 2009 to April 2010. Serum RECAF, total PSA (tPSA) and free PSA (fPSA) concentrations were measured. The results were correlated with histopathologic findings using the Mann-Whitney U test and Kruskal-Wallis χ^2 test.

Results. The median RECAF concentration was 5.34 U/L in the benign pathology group of patients and 4.72 U/L in the malignant pathology group. The difference was not statistically significant. RECAF density, tPSA and fPSA concentrations and tPSA density were significantly different between the benign and malignant pathology groups ($p = 0.033$, $p = 0.000$, $p = 0.002$ and $p = 0.000$, respectively). RECAF concentration and RECAF density did not differ significantly in the subgroups of PCa patients stratified according to Gleason score, predominant primary Gleason grade or maximum primary Gleason grade, but in predominant secondary Gleason grade and maximum secondary Gleason grade, significant differences were found ($p = 0.007$ and $p = 0.004$, respectively).

Conclusions. The results of the study did not confirm the RECAF tumor marker as an alternative way to discriminate between groups of patients with benign prostatic conditions and PCa, and its concentration and density do not differ among PCa histopathologic groups.

Key words: prostate cancer, histopathology, prostate-specific antigen, tumor marker, α-fetoprotein receptor

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In the last decade, prostate cancer (PCa) has become the most frequently diagnosed cancer among all solid tumors in men in developed countries.[1] Its increased incidence is the result of widespread screening programs and improved awareness of the disease among the general population. The discovery of the serum tumor marker prostate-specific antigen (PSA) and its adoption in clinical practice has lowered the stage of newly discovered PCa.[2] PSA is organ specific but not disease specific; it can be elevated in benign prostatic hyperplasia (BPH), prostatitis, as well as in PCa.[3] PSA values in BPH and prostatitis often resemble PSA levels in the early stages of PCa, which is a curable disease.

A diagnosis of PCa is confirmed by a transrectal ultrasound (TRUS)-guided biopsy of the prostate indicated by increased PSA levels, a suspect digital rectal examination (DRE), or suspicious areas detected on a TRUS; however, up to 60% of prostate biopsy procedures still prove to be negative using these criteria.[4–6] Several modifications of serum PSA value have been proposed to improve its specificity in the early detection of PCa, for example the ratio of free PSA (fPSA) to total PSA (%fPSA), PSA density, PSA velocity and doubling time and detection of PSA isoforms. However, their use in clinical practice is limited.[3] Other molecules that may be useful as PCa markers are under investigation. Of these, prostate cancer antigen 3 (PCA3) is the most extensively studied and has already been proposed for clinical use due to its slightly better performance compared with tPSA.[7,8]

The α-fetoprotein receptor (RECAF) is an oncofetal antigen present in high concentrations during fetal growth and development; however, its concentration drops to very low levels after birth and normally remains low even in adult life.[9,10] It has been suggested that RECAF concentrations increase in some malignant diseases (e.g., teratocarcinoma, hepatocellular, breast, lung, prostate, ovary and gastric carcinoma), whereas in benign tumors, RECAF levels do not appear elevated.[11–13]

Moro et al. studied the clinical usefulness of RECAF in the diagnostics of BPH and PCa, and they reported a sensitivity of 99% and a specificity of 95%.[11] However, no correlation to PSA concentration, histopathologic grading (Gleason grade), or the T stage of PCa was made.

The aim of the present study was to evaluate the RECAF tumor marker in the diagnostics of benign prostatic diseases and PCa and to compare it with PSA, an established clinical marker. To the authors' knowledge, this is the first study to report on correlations between RECAF and PSA and the histopathologic grading of PCa.

Material and methods

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia, and informed consent was obtained from each participant. This was a single-center study. The patients included in the study had been routinely scheduled for a TRUS-guided biopsy of the prostate by the referring urologist due to an elevated PSA concentration, suspect DRE or both. The exclusion criteria for the study were: a history of previous malignant disease, an indwelling urinary catheter or a previous positive biopsy. Any previous medical therapy for the treatment of lower urinary tract symptoms was recorded. The inclusion of the patients in the study and the TRUS-guided biopsies were performed by two certified urologists. Consecutive patients scheduled on a date on which 1 of the 2 participating urologists was performing biopsies were considered and included, if the inclusion criteria were met. While patients were scheduled for TRUS-guided biopsies up to 3 months in advance, the urologist performing the TRUS-guided biopsies was scheduled on day-to-day basis, so the patients included were a random sample. A total of 64 patients were prospectively enrolled in the study from January 2009 to April 2010. Other certified urologists and residents were performing TRUS-guided biopsies at the time of the study, but their patients were not included in the study.

Detection methods

Blood samples for analysis were obtained from each patient just before the TRUS-guided biopsies. Full blood count, serum electrolytes, alkaline phosphatase, tPSA, fPSA and RECAF markers were determined from the samples. If a patient had been taking a 5α-reductase inhibitor for over 6 months, the tPSA and fPSA concentrations measured were doubled for the statistical analysis.

The concentration of serum RECAF was measured with a competitive chemiluminescence immunoassay (CLIA) by BioCurex Inc. (Richmond, BC, Canada). The test was a solid-phase competitive immunoassay using microwell plates in which a constant amount of RECAF-acridinium competed with the RECAF in the serum sample to bind to the monoclonal antihuman RECAF antibody immobilized in the solid phase. The amount of labeled RECAF bound to the solid phase was inversely proportional to the amount of RECAF in the sample.

PSA measurements were performed on a LIAISON CLIA analyzer (DiaSorin, Saluggia, Italy) with the LIAISON tPSA and LIAISON fPSA assays. The method for quantitative determination of tPSA and fPSA is a sandwich CLIA. The light signal, and hence the amount of isoluminol-antibody conjugate, is measured by a photomultiplier as relative light units and indicates the concentration of tPSA or fPSA present in the samples.

Before each biopsy, the clinical stage according to the DRE was noted, and the volume of the prostate was measured by TRUS examination. The TRUS-guided prostate biopsies were performed using the proto-

Table 1. Patient characteristics stratified according to histopathologic results

Patient characteristics	Benign group median (interquartile range)	Malignant group median (interquartile range)	Mann-Whitney U (sig.)
Number of patients	35	29	
Age (years)	63.0 (59.0–69.0)	71.0 (61.5–76.5)	333.5 (p = 0.019)
Prostate volume (mL)	46.0 (38.0–56.8)	30.0 (23.0–40.1)	223.5 (p = 0.000)
Blood leukocytes (10^9/L)	7.2 (5.9–8.2)	6.9 (6.2–7.6)	476.5 (p = 0.676)
Hemoglobin level (g/L)	150.0 (142.0–155.0)	152.0 (140.5–158.0)	475.0 (p = 0.661)
Blood thrombocytes (10^9/L)	210.0 (176.0–262.0)	230.0 (207.0–288.5)	392.0 (p = 0.119)
Serum alkaline phosphatase (µkat/L)	1.100 (0.940–1.300)	1.80 (0.995–1.260)	496.5 (p = 0.882)

Table 2. A comparison of median RECAF concentration, median PSA concentration and derived parameters between benign and malignant groups of patients

Tumor marker	Benign group median (interquartile range)	Malignant group median (interquartile range)	Mann-Whitney U (sig.)
Number of patients	35	29[1]	
RECAF concentration (U/l)	5.34 (3.87–9.70)	4.72 (3.86–9.41)	472.5 (p = 0.637)
RECAF density (U/L/cm^3)	0.1187 (0.0734–0.2167)	0.1995 (0.1344–0.3451)	349.0 (p = 0.033)
tPSA concentration (µg/L)	3.57 (2.40–5.10)	7.71 (4.58–13.50)	197.0 (p = 0.000)
fPSA concentration (µg/L)[1]	0.540 (0.380–0.960)	0.895 (0.605–1.390)	270.0 (p = 0.002)
tPSA density (µg/L/cm^3)	0.0795 (0.0529–0.1020)	0.2658 (0.1199–0.5195)	137.0 (p = 0.000)
Free to total PSA ratio (%)[1]	18.5 (10.2–24.2)	13.0 (8.0–18.9)	363.0 (p = 0.079)

[1] Due to missing data, the number of patients in this group in rows fPSA and free to total PSA ratio is 28; RECAF – α-fetoprotein receptor; tPSA – total PSA; fpsa – free PSA.

col established in the authors' institution, targeting the lateral parts of the peripheral zone of both prostatic lobes.

The biopsy cores were analyzed in the histopathology laboratory of the Institute for Pathology at the Medical Faculty of Ljubljana University (Slovenia). The results were divided into benign and malignant categories and further subclassified as BPH, inflammation (prostatitis), high-grade prostatic intraepithelial neoplasia (HGPIN), suspected PCa and confirmed PCa. In the PCa reports, the Gleason grades were noted.

Statistical analysis

The data were analyzed using SPSS software (v. 21.0, IBM Corp., Armonk, USA). The measured concentrations of RECAF and PSA and their derived values were compared using the Mann-Whitney U test for the 2 groups of patients. The Kruskal-Wallis χ^2 test was used to compare the values of more than 2 groups of patients. The Kendall τ-b test was used to calculate the statistical correlation between PSA and RECAF concentrations and RECAF density.

Results

Complete data was obtained from 63 out of the 64 patients; in 1 patient, the fPSA concentration was not measured. Table 1 shows a comparison of general patient data stratified into benign and malignant pathology groups, in which the benign group consisted of the patients whose pathology report diagnosed BPH, prostatitis or HGPIN, and the malignant group consisted of patients with either a suspicious report for PCa or confirmed PCa.

The median RECAF concentration was 5.34 U/L (3.87–9.70) in the benign pathology group and 4.72 U/L (3.86–9.41) in the malignant pathology group. The difference was not statistically significant (Mann-Whitney U test = 472.5; p = 0.637). However, when RECAF concentration was normalized to prostate volume (RECAF density), a significant difference was found between the 2 pathology groups (p = 0.033). The tPSA and fPSA concentrations and tPSA density were also significantly different between the benign and malignant pathology groups, whereas %fPSA was not (Table 2).

The group of patients diagnosed with PCa was further stratified according to the following histologic pa-

rameters: Gleason score (Gsum), predominant primary Gleason grade (Gprim), predominant secondary Gleason grade (Gsec), maximum primary Gleason grade (maxGprim), and maximum secondary Gleason grade (maxGsec), where predominant Gleason grade refers to the predominant Gleason grade reported by a pathologist after considering all the biopsy cores, and maximum grade refers to the highest Gleason grade found in any of the biopsy cores. The median RECAF concentrations, RECAF densities, tPSA concentrations, fPSA concentrations, PSA densities and %fPSA were statistically compared among these subgroups. Out of 29 patients diagnosed with PCa, 28 had complete histopathologic data reported; in 1 patient the exact pathologic grading could not be determined due to the small amount of PCa in his biopsy cores (Table 3).

No statistically significant correlations were found between tPSA and fPSA concentrations on one hand, and RECAF concentration and density on the other hand (Table 4).

Positive statistically significant correlations were found between the age of the patient and tPSA and fPSA con-

Table 3. A comparison of median RECAF concentration, median total and free PSA concentration and derived parameters between histopathologic subgroups in the patients with prostate cancer

Gleason category	Gleason value	RECAF concentration (U/L)	RECAF density (U/L/cm^3)	tPSA concentration (μg/L)	fPSA concentration (μg/L)	tPSA density (μg/L/cm^3)	Free to total PSA ratio (%)
no. of patients		28	28	28	271	28	271
Gsum	6	9.42	0.28	4.42	0.62	0.152	18.6
	7	5.105	0.215	8.005	0.895	0.414	11.4
	8	8.025	0.324	10.94	0.90	0.361	9.0
	9	4.42	0.123	15.45	1.78	0.558	13.4
	Kruskal Wallis χ^2 (sig.)	6.539 (p = 0.088)	6.850 (p = 0.077)	12.057 (p = 0.007)	9.859 (p = 0.020)	9.389 (p = 0.025)	3.496 (p = 0.321)
Gprim	3	6.245	0.206	5.395	0.765	0.199	14.7
	4	4.42	0.168	13.50	1.60	0.558	11.0
	Mann Whitney U (sig.)	68.000 (p = 0.194)	83.000 (p = 0.546)	34.000 (p = 0.004)	35.000 (p = 0.009)	29.000 (p = 0.002)	69.000 (p = 0.348)
Gsec	3	9.29	0.336	5.57	0.68	0.214	18.0
	4	6.245	0.176	6.445	0.815	0.255	11.5
	5	4.32	0.103	15.40	1.57	0.454	11.9
	Kruskal Wallis χ^2 (sig.)	4.555 (p = 0.103)	10.063 (p = 0.007)	9.199 (p = 0.01)	8.057 (p = 0.018)	4.356 (p = 0.113)	2.216 (p = 0.330)
maxGprim	3	0.6245	0.206	5.395	0.765	0.199	14.7
	4	4.245	0.168	13.50	1.60	0.558	11.0
	5	4.52	0.143	10.81	1.40	0.415	23.4
	Kruskal Wallis χ^2 (sig.)	1.694 (p = 0.429)	0.908 (p = 0.635)	9.234 (p = 0.010)	6.894 (p = 0.032)	10.990 (p = 0.004)	0.995 (p = 0.608)
maxGsec	3	0.929	0.336	5.57	0.68	0.214	18.0
	4	0.595	0.205	6.47	0.87	0.281	11.0
	5	4.145	0.099	14.55	1.78	0.447	13.4
	Kruskal Wallis χ^2 (sig.)	4.802 (p = 0.091)	11.102 (p = 0.004)	7.784 (p = 0.020)	8.219 (p = 0.016)	3.136 (p = 0.208)	2.136 (p = 0.344)

[1] Due to missing data, the number of patients in this group in columns fPSA and free to total PSA ratio is 27; Gsum – Gleason score; Gprim – predominant primary Gleason grade; Gsec – predominant secondary Gleason grade; maxGprim – maximum primary Gleason grade; maxGsec – maximal secondary Gleason grade; RECAF – α-fetoprotein receptor; tPSA – total PSA; fPSA – free PSA.

Table 4. Statistical correlation between PSA and RECAF

Kendall τ-b (sig.)	RECAF concentration	RECAF density
Total PSA	-0.049 (0.570)	0.074 (0.388)
Free PSA	0.017 (0.840)	-0.034 (0.691)

RECAF – α-fetoprotein receptor; tPSA – total PSA; fPSA – free PSA.

centrations and PSA density, but no statistically significant correlations were found between the age of the patient and RECAF concentration and density (Table 5).

Discussion

This study focused on a comparison of novel tumor marker RECAF vs PSA, a tumor marker that has been established for PCa for 2 decades.[2] A previous study reported a sensitivity of 99% and specificity of 95% for RECAF.[11] However, the present study found no statistically significant difference in RECAF concentration between the benign and malignant pathology groups. Consequently, the sensitivity and specificity of the RECAF tumor marker were not determined. The median RECAF concentration was lower in the patients with PCa than in the benign pathology group. Since it has been suggested that RECAF, as an oncofetal protein, is expressed in large quantities in physiologic and pathologic states of high cell division and turnover rates, such as fetal development and some cancers, higher concentrations of RECAF in the PCa patients than in the benign pathology group were expected.[10–13] On the other hand, the median RECAF density was almost 1.7 times higher in the patients with PCa than in the group with benign conditions, which was a statistically significant difference. It should be noted that the median prostatic volume was almost 1.6 times lower in the malignancy group than in the benign group, possibly introducing a bias. A much larger difference in RECAF density was expected, because RECAF concentrations in benign conditions have been reported to be nearly the same as in healthy men, suggesting a very low concentration in benign prostatic conditions.[11] In contrast, the PSA measurements in the present study showed significantly higher tPSA and fPSA concentrations and tPSA density in the group of patients with PCa when compared with the benign group.

The age of the patient is a proven risk factor for PCa. The present study has shown a positive statistical correlation between age and tPSA, fPSA and PSA density, which were higher in the group of PCa patients, who were significantly older than those in the benign group. No such correlation was found for RECAF concentration or RECAF density. The positive correlation between PSA density and age underscores the fact that in older men, PSA produced per volume of prostatic tissue increases due to PCa and not due to benign prostatic enlargement.

Generally, tPSA concentration is higher in PCa patients with a higher Gsum.[14,15] The results of the present study show significantly higher median tPSA and fPSA concentrations in the subgroups of patients with increasing Gsum, Gprim, Gsec and maxGsec. Similarly, tPSA density also significantly increases in patients with increasing Gprim, implying higher prostatic cell division and turnover rates in patients with higher grade cancer. In contrast, RECAF concentration and RECAF density do not differ significantly in the subgroups of patients stratified according to Gsum, Gprim, and maxGprim; it was only in the subgroups with increasing Gsec and maxGsec that significant differences in RECAF density were found. Median RECAF concentrations decrease with increasing Gprim, Gsec, and maxGsec; median RECAF density decreases with increasing Gprim, Gsec, maxGprim, and maxGsec, even though one would expect that a higher rate of cell division and turnover in higher grade cancers would be reflected in higher RECAF concentration and density.

The results of this study did not confirm that higher tPSA and fPSA concentrations imply higher RECAF concentration and RECAF density, because no statistically significant correlations were found in the entire study group. As this study is the first to report on the RECAF marker in relation to the histopathologic grading of PCa and correlations to PSA, there are no other studies to compare the results with.

In conclusion, the results of the present study did not show RECAF to be an alternative tumor marker for discrimination between groups of patients with benign prostatic conditions and PCa. Its concentration and density do not correlate with either tPSA or fPSA, which are established markers for PCa and which differ significantly between the benign and malignant pathology groups of patients in the study. In contrast to tPSA and fPSA, RECAF concentration does not significantly differ between histopathologic subgroups according to Gsum, Gprim, and Gsec grades or maxGprim and maxGsec grades. RECAF density is, paradoxically, lower in higher-end Gleason grade and maxGsec grade subgroups. To elucidate the issue of the RECAF marker in different histopathologic groups, it is necessary to conduct further studies including histopathologic staining of prostate biopsy cores using a tissue-section staining kit approved by the US Food and Drug Administration.[11]

Table 5. Statistical correlation between PSA and RECAF with the age of the patient

Kendall τ-b (sig.)	tPSA	fPSA	PSA density	RECAF concentration	RECAF density
Age	0.245 (0.005)	0.306 (0.001)	0.213 (0.015)	-0.149 (0.088)	-0.015 (0.866)

RECAF – α-fetoprotein receptor; tPSA – total PSA; fPSA – free PSA.

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Usefulness of carcinoembryonic antigen in the diagnosis of small cell lung cancer combined with adenocarcinoma

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Small cell lung cancer (SCLC) includes pure SCLC and SCLC combined with other pathologies (C-SCLC). C-SCLC accounts for about 28% of all SCLCs subjected to surgical resection, but only about 1–3% of C-SCLCs are detected by biopsy. Since less than 5% of SCLC patients are eligible for surgery, it is necessary to develop alternative methods for the detection of C-SCLC.

Objectives. We determined whether serum carcinoembryonic antigen (CEA) levels, which are usually elevated in lung adenocarcinomas, could be used to differentiate between pure SCLC and SCLC combined with adenocarcinoma.

Material and methods. We reviewed the records of 41 SCLC patients (35 with pure SCLC, 6 with C-SCLC) who underwent surgical resection between 2000 and 2014 in Zhejiang Cancer Hospital. Their preoperative serum CEA levels were noted, and the relationship between CEA level and the type of SCLC was analyzed.

Results. Serum CEA levels >6ng/mL were found more frequently in C-SCLC patients than in pure SCLC patients ($p = 0.031$). No such difference was observed when a CEA cut-off of 5ng/mL was used ($p = 0.316$).

Conclusions. A preoperative serum CEA of >6ng/mL may be used as a reference in the diagnosis of SCLC combined with adenocarcinoma.

Key words: diagnosis, small cell lung cancer (SCLC), carcinoembryonic antigen (CEA), adenocarcinoma

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Lung cancer is one of the most common malignant tumors and has the highest mortality rate of any cancer. Small cell lung cancer (SCLC) is a highly aggressive and lethal type of cancer in humans and accounts for approx. 13% of all cases of lung cancer.[1] SCLC is sensitive to chemotherapy and radiotherapy; however, long-term survival is low, and the majority of patients eventually develop progressive disease. Moreover, there is a high rate of relapse, even among patients who have achieved a complete response. Combined SCLC (C-SCLC) is a special histological type of SCLC, which presents different tumor molecular characteristics from pure SCLC.

Although the World Health Organization (WHO) considers C-SCLC to be a subset of SCLC, C-SCLCs can have many features of non-small cell lung cancer (NSCLC).[2] Recent research has shown that, although patients with C-SCLC might have a similar prognosis as those with pure SCLC, C-SCLC patients who undergo surgery have a better overall survival rate than pure SCLC patients who undergo surgery.[2,3] The treatment of C-SCLC can also be different from that of pure SCLC.[4]

Finding an appropriate method to detect and diagnose C-SCLC is difficult. It is known that serum carcinoembryonic antigen (CEA) is usually elevated in patients with lung adenocarcinoma.[2] However, at present, no study has examined serum tumor markers as a reference for diagnosing C-SCLC. To explore the possibility of using serum CEA as a supplementary diagnostic test for SCLC combined with adenocarcinoma, we retrospectively analyzed the preoperative serum CEA levels in 35 patients with pure SCLC and 6 patients with SCLC combined with adenocarcinoma. All patients had undergone surgery at Zhejiang Cancer Hospital (Hangzhou, China), and the surgical specimens of all patients had been examined.

Material and methods

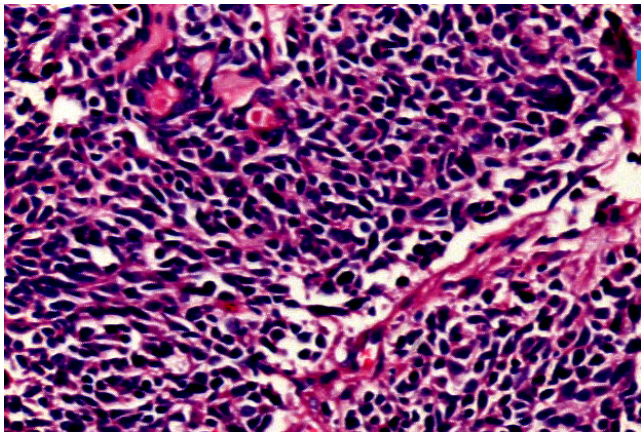
Patient characteristics

Our study involved 35 patients with pure SCLC and 6 consecutive patients with SCLC combined with adenocarcinoma (C-SCLC) who were admitted to Zhejiang Cancer Hospital between January 2000 and May 2014. This study was approved by the ethics committee of Zhejiang Cancer Hospital. SCLC staging was performed in all patients according to the 7th edition of the TNM classification for lung cancer. The preoperative serum CEA levels were retrospectively collected. In all patients, the histological diagnosis was based on the standard criteria defined in the WHO classification. Among the C-SCLC patients, 66.7% were men, 33.3% were aged >65 years, and 66.7% were heavy smokers. Two patients had stage IB disease, and 4 had stage IIIA disease. Two of the patients were non-smokers, and 4 were heavy smokers (Table 1, Fig. 1).

Table 1. Clinical characteristics of 35 patients with pure SCLC and 6 patients with SCLC combined with adenocarcinoma

Variables	Total	Pure SCLC n (%)	SCLC combined with adenocarcinoma n (%)	p-value
Age (years).				
<65	36	32 (91.4%)	4 (66.7%)	0.598
≥65	5	3 (8.6%)	2 (33.3%)	
Sex.				
female	7	5 (14.3%)	2 (33.3%)	0.567
male	34	30 (85.7%)	4 (66.7%)	
Smoking history.				
light	4	4 (11.4%)	0	0.635
mild	5	4 (11.4%)	0	
heavy	23	20 (57.1%)	4 (66.7%)	
never	9	7 (20.1%)	2 (33.3%)	
Pathological stages.				
I	11	9 (25.7%)	2 (33.3%)	0.522
II	8	8 (22.9%)	0	
III	22	18 (51.4%)	4 (66.7%)	
IV	0	0	0	

Fig. 1. Patient (70-year-old man) with stage IIIA pT2N2M0 SCLC combined with adenocarcinoma



Pathological result was detected using H&E staining, showing SCLC combined with adenocarcinoma cell lung cancer. ALK(D5F3)(-), ALK-NC(-), CK5/6(-), CK7(-), Napsin A(-), P63(-), TTF1(+). All images with original magnification ×200 and H&E stained unless otherwise indicated.

Statistical analysis

Two threshold values of CEA, 5ng/mL and 6ng/mL, were adopted to distinguish C-SCLC from pure SCLC. The data was analyzed using version 15.0 of the SPSS software package. The χ^2 test was used in univariate analyses, and corrections were done when there were less than 5 cases. Statistical significance was indicated at p-values of <0.05.

Results

Different Serum CEA levels between C-SCLC and pure SCLC patients

Most of our patients were men, older than 65 years, and had a history of heavy smoking. The clinical characteristics of the C-SCLC patients did not significantly differ from those of the SCLC patients. Preoperative serum CEA levels >6ng/mL were more frequently observed in C-SCLC patients than in pure SCLC patients (p = 0.031). No such difference was observed in the case of CEA levels >5ng/mL (p = 0.316; Table 2).

Table 2. Serum CEA level in pure SCLC patients and SCLC combined with adenocarcinoma patients

CEA (ng/mL)	Pure SCLC n (%)	SCLC combined with a denocarcinoma n (%)	p-value
≤5	27 (77.1%)	3 (50%)	
>5	8 (22.9%)	3 (50%)	0.316
≤6	32 (91.4%)	3 (50%)	
>6	3 (8.6%)	3 (50%)	0.031

Discussion

SCLC is a highly aggressive and lethal type of cancer in humans. Although there has been a modest, statistically significant improvement in 2- and 5-year survival rates over the last 30 years, the outcomes of SCLC remain extremely poor.[1] Chemotherapy is the cornerstone of therapy for SCLC. By 2002, the proportion of SCLC had decreased to approximately 12.95% of all lung cancers.[1,5] C-SCLC has been reported to account for 1–3.2% of all SCLC cases.[6,7] C-SCLC is defined by the WHO as SCLC combined with an additional component that consists of any of the histological types of NSCLC, usually adenocarcinoma, squamous cell carcinoma, or large cell carcinoma and less commonly spindle cell or giant cell carcinoma. In the case of large cellcarcinoma, an arbitrarily chosen cut-off of at least 10% large cell carcinoma is required for the diagnosis of C-SCLC.

Surgical specimens reflect the clinic pathological status, and the specimens of a high percentage of SCLC patients (28%) show an additional NSCLC component.[8] Due to the presence of crush artifacts and/or the limited availability of biopsy specimens, the possibility of detecting an NSCLC component in SCLC on histology is low. Thus, many cases of C-SCLC may be missed during the examination of biopsy specimens from SCLC patients. With improvements in diagnostic methods, more and more C-SCLC could be detected in recent years.[8] In our study, all SCLC patients underwent surgery before chemotherapy. All specimens in our study were surgical specimens, which are relatively rare and are very informative.

The 7th edition of the TNM classification has also been cited in the National Comprehensive Cancer Network (NCCN) guidelines for SCLC (2015 v. 1). T1–2N0M0 SCLC patients have been reported to account for less than 5% of all SCLC patients.[9] Among SCLC patients, only those with cancers classified as T1–2N0M0 are eligible for surgical treatment; in contrast, surgery may be used to treat NSCLC patients in stages IA, IB, IIA, IIB, and IIIA. Thus, few SCLC patients are eligible for surgery. Complete preoperative assessment is required before surgery for SCLC to exclude the presence of nodal involvement. In stage I SCLC, surgery always must be followed by adjuvant chemotherapy, while in stages II and III, surgery must be planned only in the context of clinical trials and after a pathologic response to the induction of chemoradiotherapy has been confirmed.[10] The incidence of brain metastasis can be reduced with prophylactic cranial irradiation.[11,12] It is difficult to diagnose C-SCLC by biopsy, and the rarity of patients with C-SCLC makes it difficult to determine the optimal management and biological characteristics of this tumor. Few studies have investigated C-SCLC, and more research should be conducted to identify the clinical features of these patients.[13]

Some studies have indicated that the clinical characteristics of C-SCLC patients do not significantly differ from those of pure SCLC patients.[6,8,14] Consistent with this, we found no significant differences in the clinical characteristics between SCLC and C-SCLC patients. Using genotypic and immunophenotypic analyses, Wagner et al. found that C-SCLC is biologically similar to SCLC.[14] However, the overall survival after surgery can differ between SCLC and C-SCLC patients.[2,3]

Serum tumor marker testing is a noninvasive, repeatable, and effective method for assisting the diagnosis of cancer. Serum CEA levels are invariably elevated in lung adenocarcinoma.[15,16] We, therefore, attempted to find a cut-off value of preoperative serum CEA level that would assist in the diagnosis of C-SCLC. As adenocarcinoma is a common additional component in C-SCLC, our research focused on evaluating the usefulness of preoperative serum CEA levels in the diagnosis of SCLC combined with adenocarcinoma.

Pretreatment CEA levels of 3–10 ng/mL have been reported in lung cancer.[15,17–19] In this study, a CEA cut-off of 5 ng/mL was not found to be useful for distinguishing SCLC combined with adenocarcinoma from pure SCLC. This may be because most of our patients had a history of cigarette smoking, and serum CEA levels are known to be elevated in smokers.[20–22] We found that a relatively higher threshold of 6 ng/mL CEA was useful for distinguishing SCLC combined with adenocarcinoma from pure SCLC.

Although the prognosis of C-SCLC is similar to that of pure SCLC, its sensitivity to chemoradiotherapy is lower than that of pure SCLC.[23] This phenomenon is attributable to the mixed NSCLC component in C-SCLC. The possibility of a tumor with combined pathologies should be considered in patients who are thought to have SCLC on the basis of limited biopsy materials, such as needle aspiration or bronchial biopsy specimens, or when the primary lesion is found to be peripherally located on chest radiography.[6] Our previous study has shown that epidermal growth factor receptor (EGFR) mutations may occur in C-SCLC, particularly when the "combined" component is adenocarcinoma.[13] More effective treatments (e.g., EGFR-tyrosine kinaseinhibitors) can be administered to patients who have SCLC combined with adenocarcinoma if a definite diagnosis is achieved.[4,24]

Conclusions

In conclusion, our retrospective study suggested a role for serum CEA level as a reference marker in the diagnosis of SCLC combined with adenocarcinoma. If SCLC patients have a serum CEA level higher than 6 ng/mL, they may have SCLC combined with adenocarcinoma and should be offered a further work-up (e.g., repeat or multi-point biopsy) in order to reach an accurate diagnosis. However, further prospective studies are required to support this conclusion.

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Oxidative stress parameters in patients with prostate cancer, benign prostatic hyperplasia and asymptomatic inflammatory prostatitis: A prospective controlled study

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Abstract

Background. The imbalance between oxidant and reductant mechanisms creates a nidus for the etiopathogenesis of several diseases. In this study, we aimed to compare the oxidative stress (OS) parameters in patients who were diagnosed with prostate cancer (pCa), benign prostatic hyperplasia (BPH) or asymptomatic inflammatory prostatitis (AIP), according to the histopathologic examination of transrectal ultrasonographic prostate biopsy and transurethral prostate resection specimens.

Objectives. In this study, we aimed to compare oxidative stress between histologically proven prostate cancer, hyperplasia and prostatitis.

Material and methods. According to histopathologic examinations, 97 patients were divided into 3 study groups: group 1: pCa (n = 30), group 2: BPH (n = 41), and group 3: AIP (n = 26). Finally, 30 patients were enrolled in a control group. MDA levels, CuZn-SOD, Se-GPx, CAT activities, and trace element levels were evaluated.

Results. A statistically significant difference between prostate cancer and other groups were documented in terms of MDA activity. Contrary to AIP, a statistically significant difference has also been encountered between BPH and the control group. Decreased CuZn-SOD enzyme levels were found in PCa and BPH patients without statistical significance. Increased CAT activity was also documented in PCa, BPH and AIP patients. No significant difference in GPX activity was documented between the groups, except BPH and control group. Trace element levels were low in the patients with prostate cancer and BPH when compared with the control group.

Conclusions. Despite the data regarding OS in PCa patients, there is a paucity of data regarding BPH and especially AIP patients. Our study revealed obvious oxidative stress in BPH and PCa patients as opposed to AIP. Assessing the oxidative stress in these patients may assist in the future prevention, diagnosis and also treatment. However, the question whether the presence of OS-related parameters and drugs could be used for the diagnosis or management of prostatic diseases, needs to be addressed in future larger and better studies with a more rational basis.

Key words: prostate cancer, oxidative stress, chronic prostatitis, benign prostatic hyperplasia, prostate biopsy

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Oxidative stress (OS) is defined as the interruption of the balance between oxidant and reductant molecules due to the excessive production of reactive oxygen species (ROS). This imbalance leads to oxidative DNA damage and performs a nidus for the etiopathogenesis of several diseases. There is plenty of data regarding the relationship between ROS and age-related pathologies such as cancer, diabetes or several degenerative disorders.[1] In regard to the prostate, the studies conducted in the last decade have demonstrated that OS is associated with prostate cancer (PCa) development, progression and response to therapy.[1,2] In the last years, a relationship between prostatic inflammation and benign prostatic hyperplasia (BPH) has also been suggested.[3] Conversely, there is a paucity of data regarding the OS parameters in the asymptomatic inflammatory prostatitis (AIP) patients.

In this prospective controlled study, we aimed to compare the OS parameters in patients who were diagnosed with prostate cancer, BPH or AIP according to the histopathologic examination of transrectal ultrasonographic prostate biopsy (TRUS-Bx) and transurethral prostate resection (TURP) specimens.

Material and methods

Subjects and study design

This prospective controlled study was approved by the Ethical Committee of Gülhane Military Medical Academy, protocol number 1491-1175-10/1539, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the participants in the study group and before the collection of blood specimens. A total of 127 patients were enrolled to the study to form 3 study groups and a control group. According to the histopathologic examination of TRUS-Bx and TURP specimens, 97 patients were divided into 3 study groups: group 1: prostate cancer (n = 30), group 2: benign prostatic hyperplasia (n = 41), and group 3: asymptomatic inflammatory prostatitis (n = 26). A final group of 30 patients with no lower urinary tract symptoms, normal PSA levels, normal digital rectal examination and no history of previous prostatitis treatment was enrolled as group 4 (control group).

Inclusion and exclusion criteria

Inclusion criteria: TRUS Bx performed on patients due to elevated serum PSA levels or abnormal digital rectal examination and TURP performed on patients due to lower urinary tract symptoms.

Exclusion criteria: Previous history of cancer treatment, presence of liver dysfunction, diabetes mellitus, heart failure or renal failure; smoking; chronic alcohol use, and oral antioxidant supplementation or any mineral supplementation at the moment of the enrollment.

Sample collection and laboratory methods

Blood samples were drawn from the antecubital vein following an overnight fast and distributed into evacuated tubes containing ethylendiaminetetraacetic acid (EDTA). All samples were centrifuged for 10 min at 4000 g and 4°C. After the plasma was separated, the buffy coat was removed and the packed cells were washed 3 times with 2 volumes of isotonic saline. Then, a known volume of erythrocytes was lysed with cold distilled water (1:4), stored in a refrigerator at 4°C for 15 min and the cell debris was removed by centrifugation (2000 g at 4°C for 10 min). Plasma samples and erythrocyte lysates were stored at –70°C until assayed. Copper and zinc-containing superoxide dismutase enzyme (CuZn-SOD), selenium-dependent glutathione peroxidase (Se-GPx) and catalase (CAT) activities were measured in the erythrocyte lysates on a UV-VIS recording spectrophotometer (UV-2100S, Shimadzu Co., Kyoto, Japan). Erythrocyte CuZn-SOD activity was measured as previously described by Eken et al.[4] The measurement of erythrocyte CuZn-SOD enzyme activity was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with INT to form a red formazan dye. CuZn-SOD activity is expressed in U/g Hb. Erythrocyte Se-GPx activity was measured as previously described by Tüzün et al. and expressed in U/g Hb.[5] Erythrocyte CAT activity was measured in hemolysates at 25°C by the method developed by Aebi.[6] The activity is expressed as KU/g Hb. Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances in erythrocyte lysates by the method previously described by Eken et al.[4] After malondialdehyde (MDA) reacted with thiobarbituric acid, the reaction product was followed spectrophotometrically at 532 nm, using tetrametoxypropane as a standard. The results are expressed as nmol/mL. An atomic absorption spectrometer with a Zeaman background correction (PerkinElmer Analyst 800, Shelton, CT 06484-4794 USA) was used to detect the trace element levels: zinc (Zn), copper (Cu), and selenium (Se) in plasma and erythrocyte samples.

Statistical analysis

Statistical Package for Social Sciences (SPSS) v. 15.0 was used as a software package for statistical evaluations. All results were presented as mean ±standard deviation (SD). Conformity to the normal distribution of variables was assessed by the Kolmogorov-Smirnov test. In order to compare the continuous variables among groups, the ANOVA test was used. Pearson's correlation analysis was used to evaluate the relationship between variables. Age-adjusted analysis of covariance was used for comparisons. The values were considered statistically significant, if the p-value was less than 0.05.

Table 1. MDA, antioxidant enzyme and trace element levels according to the groups

	PCa (n = 30)	BPH (n = 41)	AIP (n = 26)	Control (n = 30)
MDA (nmol/mL)	12.26 ±3.08	7.69 ±2.41	6.66 ±1.73	5.95 ±1.11
CuZn-SOD (U/g Hb)	615.29 ±142.28	575.99 ±110.31	647.80 ±39.71	686.57 ±95.61
CAT (KU/g Hb)	62.21 ±10.67	53.31 ±9.67	57.44 ±11.68	50.41 ±9.17
Se-GPx (U/g Hb)	5.18 ±1.18	4.87 ±0.91	5.45 ±0.65	5.83 ±1.17
Cu (µg/mL)	0.56 ±0.11	0.51 ±0.08	0.58 ±0.04	0.63 ±0.08
Zn (µg/mL)	7.89 ±1.53	7.22 ±1.15	8.10 ±0.62	8.68 ±1.11
Se (ng/mL)	98.96 ±19.21	91.08 ±14.16	101.60 ±5.11	111.41 ±11.67

PCa – prostate cancer; BPH – benign prostatic hyperplasia; AIP – asymptomatic inflammatory prostatitis; MDA – malondialdehyde; CuZn-SOD – copper and zinc-containing superoxide dismutase enzyme; Se-GPx – selenium-dependent glutathione peroxidase; CAT – catalase; Cu – copper; Zn – zinc; Se – selenium.

Results

Thirty newly diagnosed men with prostate cancer, 41 men with benign prostatic hyperplasia, 26 men with asymptomatic inflammatory prostatitis, and 30 control subjects were enrolled in the study. Mean patient ages were 65.16 ±8.40, 68.65 ±7.35, 64.76 ±8.09 and 53.93 ±5.41 years in prostate cancer, benign prostatic hyperplasia, asymptomatic inflammatory prostatitis and control groups, respectively. Total serum PSA levels were significantly high in the prostate cancer group when compared to other groups ($p < 0.001$). On the other hand, there was no statistical difference between the groups in terms of height, weight, body mass index and hemoglobin levels ($p > 0.05$).

MDA, antioxidant enzyme and trace element levels according to the groups are summarized in Table 1, and the comparison of the groups according to the OS parameters is shown in Table 2. A statistically significant difference between prostate cancer and other groups were documented in terms of MDA and catalase activity ($p < 0.05$). The only exception was between prostate cancer and AIP group catalase activity. Prostate cancer patients had lower CuZn-SOD levels than control group patients but the difference was not statistically significant ($p \geq 0.05$). BPH group had a lower CuZn-SOD, Se-GPx activity and a higher MDA activity when compared with the control group and all differences were statistically significant, except MDA activity ($p < 0.05$). CAT activity was the second parameter to be found as statistically insignificant. The AIP group had comparable results with those of the control group. CAT activity was found to be significantly high in the AIP group ($p < 0.05$).

Trace element levels were significantly low in patients with prostate cancer and BPH when compared with the control group, and the differences were statistically significant. The only exception was the Zn levels between the group with prostate cancer and the control group. Again, there was no statistical significance between the AIP group and the control group in terms of trace elements.

Table 2. The comparison of the groups according to OS parameters

Parameters	PCa & BPH	PCa & AIP	PCa & control	BPH & AIP	BPH & control	AIP & control
MDA	<0.001	<0.001	<0.001	0.454	0.068	1
CuZn-SOD	0.762	1	0.130	0.05	0.004	1
CAT	0.003	0.503	0.001	0.692	1	0.137
Se-GPx	1	1	0.184	0.169	0.012	1
Cu	0.099	1	0.043	0.015	<0.001	0.267
Zn	0.119	1	0.135	0.022	<0.001	0.602
Se	0.128	1	0.016	0.021	<0.001	0.116

PCa – prostate cancer; BPH – benign prostatic hyperplasia; AIP – asymptomatic inflammatory prostatitis; MDA – malondialdehyde; CuZn-SOD – copper and zinc-containing superoxide dismutase enzyme; Se-GPx – selenium-dependent glutathione peroxidase; CAT – catalase; Cu – copper; Zn – zinc; Se – selenium; $p < 0.05$ considered as statistical significance.

Discussion

Oxidative stress is the interruption of the balance between oxidant and reductant mechanisms and the excessive production of ROS. This imbalance leads to oxidative DNA damage and creates a nidus for the etiopathogenesis of several diseases.[1] In relation to the prostate, there is plenty of data regarding the relationship between ROS species, OS and age-related pathologies, such as PCa, BPH and AIP.[1–3] In our prospective controlled study, we aimed to investigated the OS parameters in patients with PCa, BPH and AIP, and compared the results with a control group. All the patients were diagnosed according to the histopathologic examination of transrectal ultrasonographic (TRUS) prostate biopsy (Bx) and transurethral prostate resection (TURP) specimens.

While MDA, hydrogen peroxide, superoxide radical or nitric oxide can be used to evaluate OS status, several endogenous antioxidant enzymes including SOD, GPx, CAT, some trace elements including Cu^{+2}, Zn^{+2}, Se^{+2}, and some molecules like vitamin E, vitamin C, transferrin and ceruloplasmin can be used in order to evaluate antioxidant capacity.[7–10] In our cohort, we analyzed MDA, SOD, GPx, CAT and Cu^{+2}, Zn^{+2}, Se^{+2} to evaluate the OS status.

MDA levels are considered to be a valuable parameter to evaluate lipid peroxidation and OS. MDA is a highly reactive aldehyde and has the potential for DNA damage, probably leading to mutagenic, genotoxic and cytotoxic effects.[11] While Dogru-Abbasoglu et al. did not find a significant difference in MDA levels in comparison to PCa and BPH patients, some other investigators documented significantly increased MDA levels in PCa patients when compared with BPH and control.[12–17] In our study, a statistically significant difference between patients with prostate cancer and other groups was documented in terms of MDA activity. On the contrary, no statistically significant difference was encountered between AIP/control and BPH/control groups.

In the literature in English, there are debatable results regarding antioxidant activity in PCa or BPH patients. While some authors documented decreased activity, others revealed no change.[18–20] In their study, Doğru-Abbasoglu et al. compared PCA and BPH patients in terms of SOD, CAT and GPx activity and showed no significant difference.[11] Aydin et al. conducted a controlled study to evaluate antioxidant activity in the patients with PCa and BPH and revealed that PCa patients had decreased CuZn-SOD enzyme levels when compared with BPH and control.[21] Jun-Fu Zhou et al. also demonstrated significantly decreased CuZn-SOD levels in chronic bacterial prostatitis (category 3), but the literature data regarding AIP patients is lacking.[22] In our study, decreased CuZn-SOD enzyme levels were found to be lower in PCa and BPH patients, but these differences were statistically significant only for BPH and control group patients.

There are also conflicting results regarding CAT and GPx activity in PCa or BPH patients. Some authors demonstrated decreased activity while others showed no difference or increased activity.[21,23–25] In their study, Biri et al. reported increased CAT and GPx activity in PCa patients when compared with BPH and control.[25] They concluded that this increase was due to a rebound effect to increased oxidative stress in order to neutralize it. Our results also revealed increased CAT activity in PCa, BPH and AIP patients when compared with the control group. The difference was statistically significant in PCa patients when compared with BPH and control group patients. But no significant difference in GPx activity was documented between groups, except BPH and control group.

Cu^{+2} and Zn^{+2} catalyze SOD enzyme and their levels are consistent with SOD levels. On the other hand, Zn^{+2} has an additional contribution to hormonal function on prostatic tissue.[26] Yan et al. hypothesized that decreased levels of Zn^{+2} are associated with increased DNA damage.[27] Christudoss et al. and Gomez et al. have found that Zn^{+2} levels were decreased in PCa patients when compared with control.[28,29] In our study, Zn^{+2} levels were decreased in PCa, BPH and AIP groups when compared with control, but a statistically significant difference was observed only between BPH and control groups. Similar results were documented also for Cu^{+2} levels but the statistical significance was seen in both PCa/control and BPH/control groups.

Se^{+2}, a trace element, is found to be protective against several malignancies with respect to several animal and cell culture studies.[30] This is considered to be due to the induction of apoptosis, which prevents cellular proliferation and has a key role for GPx enzyme activity.[31–33] There are some studies showing decreased levels in PCa and BPH patients, in contrast to some showing no difference.[34–37] Our results revealed statistically decreased Se^{+2} levels in PCa and BPH groups when compared with control group.

Conclusions

Oxidative stress, interruption of the balance between oxidant and reductant mechanisms, can lead to several prostatic diseases. Although there is plenty of data regarding OS in PCa patients, there is a lack of data regarding BPH and especially AIP patients. Our prospective controlled study has its unique advantage of evaluating several OS parameters in these patients and showed obvious oxidative stress in BPH and PCa patients as opposed to AIP. Assessing the oxidative stress in these patients may assist in the future prevention, diagnosis and also treatment. Despite our encouraging results, whether the presence of OS-related parameters and drugs could be used for the diagnosis or management of prostatic diseases is something that needs to be addressed in several future larger and better studies with a more rational basis.

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Chest HRCT findings in patients with primary Sjögren's syndrome

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Abstract

Background. Pulmonary manifestations (PMs) in primary Sjögren's syndrome (pSS) are among the most frequent extraglandular complications, with reported prevalence varying widely (9–75%), depending on the methods of detection.

Objectives. The aim of this study was to assess the incidence of PMs in pSS and to determine the factors predisposing to the occurrence of this complication.

Material and methods. The study group consisted of 68 patients with pSS. Among the patients who were possibly affected by PMs, chest High Resolution Computed Tomography (HRCT) was performed.

Results. In the group of all patients afflicted with pSS, 30 people indicated the need to expand medical imaging via chest HRCT scan. (The most frequent reason, in 80% of patients, was persistent, dry cough periodically waking up patients at night). The chest HRCT scan revealed lung tissue changes in the course of 29% of all examined patients (of 68). No correlation was found between the occurrence of HRCT changes and the age of patients ($p = 0.8$), increased CRP > 5 mg/1 ($p = 0.1$) or ESR > 20 mm/h ($p = 0.9$), focus score ($p = 0.8$), leucopenia ($p = 0.5$), RF value ($p = 0.3$), gamma globulin value ($p = 0.5$), intensity of eye and oral cavity dryness ($p = 0.6$; 0.3), and smoking cigarettes. Additionally, no correlation was found between more frequent occurrences of antibodies anti-SSA, anti-SSB or anti-Ro52 and HRCT changes ($p = 0.3$; 0.07; 0.4). Pertaining to the clinical signs, HRCT changes occurred more often only in patients suffering from peripheral arthritis ($p < 0.01$).

Conclusions. PM is a frequent symptom of pSS. A factor predisposing to the development of changes in the respiratory system was not found. Changes in HRCT occur more frequently in patients with peripheral arthritis.

Key words: chest HRCT, primary Sjogren's syndrome, pulmonary manifestation

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Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease with symptoms occurring in many organs, specifically in the salivary glands, lacrimal glands and musculoskeletal system. Symptoms are often weakly expressed resulting in a mean diagnostic delay of 6–7 years.[1] Lymphocytic infiltrates characteristic of pSS may be localized in the respiratory tract. The prevalence of respiratory symptoms in pSS varies a lot (9–75%), depending on the publications and assessment methods (clinical symptoms, chest X-ray, computed tomography).[2] High Resolution Computed Tomography (HRCT) is a relatively noninvasive method and is currently the most important method for detecting early lung parenchymal abnormalities and reduced lung function. Common HRCT findings of pSS in the lungs include ground-glass attenuation, bronchiectasis, a reticular pattern and honeycomb appearance.[3] The presence of honeycombing was associated with increased mortality in pSS. There was a fourfold increased risk of dying after 10 years of the disease among patients with lung involvement compared with those without lung involvement in a Norwegian population.[4]

Changes in the respiratory tract in pSS can occur as variable symptoms, which was considered in the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI).[5] It consists of the subjective and objective symptoms including persistent dry cough, bronchial involvement, shortness of breath, radiographic abnormalities on radiography or in chest HRCT and abnormal lung function tests (70% > DL_{CO} ≥ 40% or 80% > FVC ≥ 60%). The pulmonary weight has a value of 5 points. A maximum total score of 15 points can be achieved. It is suggested that a score of 5 points is associated with moderate pSS activity and > 14 points with shorter survival.[6,7]

The aim of this study was to assess the incidence of pulmonary manifestations in pSS and to determine factors predisposing to this complication.

Material and methods

The study group consisted of 68 patients (66 women and 2 men) with a diagnosis of pSS on the basis of the American-European criteria of Sjögren's Syndrome Classification from 2002[8], who were treated in the Department of Rheumatology and Internal Medicine, Wroclaw Medical Hospital, in the years 2010–2014. Written informed consent was obtained from each patient before the study. The study was accepted by the Commission of Bioethics at Wroclaw Medical University (no. 357/2010). The median age of patients was 51 (19–82) years. The median time between the onset of first symptoms and diagnosis of pSS was 7.5 years; among patients with changes in the respiratory system, the average time was 7 years and it was the shortest in comparison to other initial symptoms: changes in major salivary glands (10 years), enlarged peripheral lymph nodes (9 years), skin changes (11 years), arthritic changes (9 years). Among the patients who were possibly affected by pulmonary manifestations, chest HRCT was performed without and with administration of contrast medium. Symptoms leading to the expanding of medical imaging by chest HRCT were dry cough not related to the infection and lasting for 3 months, dyspnea, decreasing stamina due to the symptoms of dyspnea or paroxysmal dry cough.

The efficacy of immunosuppressive therapy was assessed on the basis of the chest HRCT performed on patients who have been receiving treatment for more than 6 months (Table 1, patient number 3, 5, 6, 8–11, 13, 15 and 16). The examination was performed with the same equipment. Additionally, all of these patients underwent basic laboratory tests including a complete circumferential blood count, concentration of C-reactive protein (CRP, nv < 5 mg/L), erythrocyte sedimentation rate (ESR, nv < 15 mm/h), value of gamma globulins marked in electrophoresis of proteins (nv 0.6–1.2 g/dL), immunological panel including rheumatoid factor (RF, nv < 14 IU/mL) and specific antibodies anti-SSA, anti-SSB and anti-Ro52 (Anti-ENA ProfilePlus1 EUROLINE, EUROIMMUN, Lübeck, Germany) as well as the assessment of lymphoid infiltrates in minor salivary glands graded on the focus score scale (0–4). The assessment of the degree of intensity of symptoms of the oral cavity and eye dryness was based on the EULAR Sjögren's Syndrome Patient Reported Index Scale (ESSPRI).[9] 0 points indicated no symptoms of dryness whereas 10 points indicated strong intensity of dryness which made everyday functioning difficult for patients. In statistical analysis, the Student's t-test, Kruskal-Wallis test and ANOVA were used to compare 3 groups of patients (with positive and without changes in chest HRCT and patients without chest HRCT). The assumed alpha significance level was 0.05, $p < 0.05$.

Results

In the group of all patients with pSS, 30 (44%, females only) indicated the need to expand medical imaging via chest HRCT scan. The symptoms leading to this examination were as follows: in 80% of cases it was constant, dry and persistent cough periodically waking up the patients at night. In 18% of cases it was the decreased stamina mainly due to coughing fits (91%). In 2% of cases, dyspnea not related to dry cough was reported. There were no changes in patients undergoing clinical chest examination. No correlation was found between the respiratory system symptoms and cigarette smoking. There were no other factors which were found to have an influence on the changes in the lungs, for example profession, older age and longer duration of pSS. In the group of patients with pSS who did not have a HRCT scan first, chest radiography was obtained, which were positive in all but 2 cases (a lung tumor and suspicion of in-

Table 1. Treatment of patients with changes in the chest HRCT

Patient No.	Treatment duration (months)	Treatment before chest HRCT	Efficacy of treatment (control chest HRCT)	Encorton (mg)	Cyclosporine (mg)	Azathioprine (mg)	Type of changes (chest HRCT)
1	<6	HQ	–	–	–	150	N/ F
2	<6	HQ,GKS	–	10	–	–	ML
3	12	HQ, CYC	stabile	2.5	150	–	N
4	<6	CHQ,CYC,GKS	–	10	–	150	N/ ML
5	6	CHQ,GKS	complete regression	7.5	150	–	GGO/ ML
6	9	n dgn	stabile	5	150	–	N
7	<6	HQ,AZA,	–	–	–	175	F
8	15	CHQ, GKS	stabile	10	–	100	F/ ML
9	12	n dgn	progression	10	–	150	GGO
10	13	n dgn	complete regression	5	–	150	F/ ML
11	13	CYC	–	2.5	–	100	GGO
12	<6	MTX	partial regression	–	–	100	F
13	12	MTX, GKS	–	5	–	150	LIP
14	<6	CHQ, GKS	stabile	–	100	–	N
15	12	HQ	progression	5	150	–	N/ F
16	21	CHQ	–	10	–	50	GGO/ ML
17	<6	CHQ	–	–	–	150	F
18	<6	n dgn	–	5	–	–	F
19	<6	GKS	–	15	150	–	LIP/ ML
20	<6	n dgn		5	–	–	F

N – nodules; F – pulmonary fibrosis (in this, GGO – ground-glass opacity image); LIP – lymphocytic interstitial pneumonia; ML – mediastinal lymphadenopathy; GKS – corticosteroids; MTX – methotrexate; HQ – hydroxychloroquine; CHQ – chloroquine; n dgn – new diagnosis; chloroquine at a dose of 250 mg per day or hydroxychloroquine at a dose of 200 mg per day were administered in the treatment of all patients except one, number 19; cyclophosphamide at a dose of 800 mg per month was administered intravenously in the treatment of patient number 2.

terstitial changes) indicating the need for HRCT scan. The chest HRCT scan in 30 patients revealed lung tissue changes in the progress of pSS in 20 of 30 patients (66%), which made up 29% of all the examined patients (of 68). The discovered changes were: nodes (30%), emphysema (40%), fibrosis (65%), lymphocytic interstitial pneumonia (10%) and enlarged mediastinal lymph nodes (35%). Emphysema changes have never exemplified a singular pathology and they have always involved the co-occurrence of nodules and pulmonary fibrosis. The mean age of patients diagnosed with chest changes seen on HRCT scan was 52 years. No correlation was found between the occurrence of HRCT changes and the age of the patients ($p = 0.8$), CRP concentration ($p = 0.3$), increased CRP concentration CRP > 5 mg/1 ($p = 0.1$), ESR ($p = 0.2$), abnormal ESR value – ESR > 20 mm/h ($p = 0.9$), intensity of infiltration in focus score ($p = 0.8$), number of leucocytes ($p = 0.7$), leucopenia < 4 × 10*9/L ($p = 0.5$), RF value ($p = 0.3$), gamma globulin value ($p = 0.5$), intensity of eye dryness ($p = 0.6$) or intensity of oral cavity dryness ($p = 0.3$). Additionally, no correlation was found between more frequent occurrence of the antibodies anti-SSA, anti-SSB or anti-Ro52 and HRCT changes ($p = 0.3$; 0.07; 0.4). According to the clinical data, HRCT changes occurred more often only in patients suffering from peripheral arthritis ($p < 0.01$). The characteristics of all patients with pSS are presented in Table 2.

Treatment of lung changes and its effectiveness: 76% of 68 patients used chloroquine (250 mg/day or hydroxychloroquine (200 mg/day, 7% azathioprine (mean

Table 2. The characteristics of all patients with pSS

Patients with pSS all study groups	With changes in chest HRCT	Without changes in chest HRCT	p-value
Number of patients 68	20	10	–
Age 51 (19–80) years (min-max)	52 (31–72)	48 (27–64)	0.78
CRP (mg/L) 2.33	2.85	1.64	0.37
ESR (mm/h) 26	37	39	0.24
γ-globulin (g/dL) 1.4	1.7	1.5	0.50
RF (IU/mL) 92	66	55	0.37
Focus score (0-4 score) 2	2	2	0.87
ESSPRI (cm):			
eye dryness 4.2	4.9	4.3	0.64
oral cavity dryness 4.4	4.7	6.1	0.29
leucopenia <4000/u (n) 29	6	4	0.57
Clinical signs (number of patients):			
skin changes 23	6	3	1.0
fatigue 28	15	8	0.27
arthralgia 48	15	9	0.14
salivary gland enlargement 33	10	7	0.29
arthritis 22	15	1	<0.001
Raynaud syndrome 1	1	0	0.47
peripheral neuropathy 8	3	2	0.72
lymphadenopathy 15	6	6	0.11

CRP – C-reactive protein; ESR – erythrocyte sedimentation rate; γ-globulin – value of gamma globulins marked in electrophoresis of proteins; RF – rheumatoid factor; ESSPRI – EULAR Sjögren's Syndrome Patient Reported Index Scale; (n) – number of patients.

125 mg/day), 20% methotrexate (mean 16.5 mg/week), 4% ciclosporin (mean 145 mg/day) and 41% prednisolone (mean 6 mg/day).

Chloroquine at a dose of 250 mg per day or hydroxychloroquine at a dose of 200 mg per day were administered in the treatment of all patients except one where ophthalmic contraindications made such therapy impossible. The details of the pharmacotherapy are presented in Table 1. Additionally, cyclophosphamide at a dose of 800 mg per month was administered intravenously in the treatment of one patient (number 2) due to vasculitis manifested by palpable purpura and ulcers of the lower extremities. There was no correlation between treatment in all 68 patients and the indication to do chest HRCT ($p = 0.7$, Student's t-test).

Discussion

T-lymphocyte infiltration localized in airways is leading to the epithelial cells damage and to the loss of their secretory function which is primarily manifested by coughing. Respiratory manifestations in pSS are heterogeneous and may include: dry cough, diffuse panbronchiolitis (DPB), bronchiectasis, nonspecific interstitial pneumonia (NSIP), idiopathic pulmonary fibrosis (IPF), cryptogenic organizing pneumonia (COP), lung cysts, nodular opacities, follicular bronchiolitis, lymphoid interstitial pneumonia (LIP), pseudolymphoma, lymphomatoid granulomatosis, lymphoma (usually of mucosa-associated lymphoid tissue type-MALT), pulmonary amyloidosis, pulmonary hypertension and pleural involvement.[11,12] The mediastinal manifestations of pSS include lymphadenopathy, thymic lymphoid hyperplasia, multilocular thymic cysts and, rarely, MALT lymphoma.[11,12] Such a wide range of manifestations may lead to significant diagnostic and therapeutic difficulties. Occasionally, a histopathological assessment of lung biopsy is needed. In HRCT scans, bronchial wall thickening (8–68%), nodules (6–29%), bronchiectasis (5–46%), air trapping (32%) and ground-glass attenuation were most frequently observed.[11–13]

According to available publications, the prevalence of respiratory manifestations in pSS is estimated to be about 9–12% and clinical features are present in about 43–75% of patients when radiology imaging such as chest X-ray, HRCT or MRI are performed.

Papiris et al. found that cough occurred in 41% of patients with pSS.[10] When HRCT was performed in all patients, the findings in the lungs were observed in up to 50% of patients; and when BAL was performed, the percentage increased to 55% in patients with pSS without clinical symptoms of lung involvement. The transformation risk of BAL changes into severe lung disease is unknown, therefore this test could not be routinely used in pSS with prognostic value. The clinical symptoms of lymphoma are frequently nonspecific and include dry cough or slowly increasing dyspnea.[10] Similar to Papiris et al. in a comparable percentage (44% of patients), there were indications to perform chest HRCT. We found that the most common indication in 80% of patients was a persistent dry cough for at least 3 months and rarely dyspnea, reduced exercise tolerance due to dyspnea or sudden attacks of dry coughing. In the group of 30 patients, in case of 20 of them (66%) changes on HRCT scans were found (29% of patients from the whole group with pSS). Pulmonary fibrosis, emphysematous changes and mediastinal lymphadenopathy were the most common findings. In the recent Norwegian study, the percentage of lung involvement on HRCT scans in patients with pSS was similar to our findings (23% of patients out of 217 with pSS).[4]

Contrary to our research, most other studies reported the presence of predictive factors for pulmonary involve-

ment in pSS (e.g., anti-SSB antibodies or hypergammaglobulinemia).[14] Hyperglobulinemia was often observed in patients with pulmonary involvement in pSS, which was not confirmed in our study.[11] Unlike our results, the risk of airway involvement increased with male gender, older age at the time of diagnosis and in smokers.[10]

Factors such as older age and longer duration of pSS may be associated with bad prognosis but this was not confirmed in our study. Palma et al. found that the quality of life was lower in patients with pSS associated with pulmonary involvement compared to patients without airway changes, according to the Medical Outcomes Study 36-Item Short-Form Health Survey Physical Functioning ($p = 0.03$). Additionally, in patients with pulmonary findings, increased mortality was observed after 10 years ($p = 0.002$, 17% vs 4.5%).[4]

Currently, the correlation between anti-SSA and anti-SSB antibodies and airway involvement is not precisely explained and the findings from various studies contradict each other.[14] Generally, the lung findings were more common when the anti-SSB antibodies were found than anti-SSA antibodies, which was not confirmed in our study, but the p-value was higher in patients with anti-SSB antibodies than with anti-SSA antibodies in our study.

Many types of cytokines, such as IL-10, IL-6 and TGF-β, INF-γ, a unique chimera-type member of the β-galactoside-binding soluble lectin family galectin-3 and lymphocytes Th1 and Th17 have been known to regulate the pathogenic process of ILD.[15–17]

Lin Yang et al. suggested the protective role of autoantibodies against interferon-γ, which significantly reduces the frequency of pulmonary fibrosis and concentration of C-reactive protein in patients with pSS. Autoantibodies against interferon-γ may become a prognostic marker of pulmonary manifestations and have a close correlation with autoimmune inflammation in pSS.[18] In patients with pSS without prior ILD, the cumulative incidence of ILD in patients with pSS was 10% (±3%) 1 year after diagnosis of pSS and increased to 20% (±4%) 5 years after diagnosis of pSS. The development of lung disease in pSS was associated with poor survival.[19] Thus, repeated pulmonary function tests and diagnostic radiology are necessary and should be the preferred methods to monitor pSS-specific organ involvement.

Pulmonary involvement in pSS may present in various forms and clinical manifestations that were included in the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI); this index includes clinical symptoms and objective tests such as persistent cough, shortness of breath on exercise, radiological or HRCT evidence of interstitial lung disease and abnormal lung function tests ($DL_{co} < 70\%$, $FVC < 80\%$).[5] Pulmonary domain weight stands at 5 points. Patients can accumulate 15 points in the case of the highly active pulmonary involvement. Five points in the ESSDAI index is considered to be a moderate activity of pSS and 14 points or more are correlated with increased mortality.[6] According to Ramos-Casals et al., the clinical domain with greatest activity during follow-up in comparison to the activity measured at diagnosis was the pulmonary part. At the beginning of the study, changes were observed only in 6% of patients and after 75 months the percentage increased to 15%. The mean total ESSDAI score for this domain was low (2–5). Older patients at the time of diagnosis (> 70 years) were less active during follow-up, but had a higher pulmonary activity score.[7]

Findings on the chest HRCT scan in our study were more common in patients with arthritis, but the group with these complications was relatively small.

For the time being, treatment of the pulmonary manifestations is undefined and is based on clinical experience. The explicit guideline (algorithm) does not exist yet. In patients with pSS and changes in the upper respiratory tract the most common symptoms are associated with dryness. Thus, muscarinic receptor agonists (pilocarpine and cevimeline) in nasal or throat sprays and humidifiers may be used in therapy. In pulmonary involvement, the treatment consists of immunosuppressive drugs such as hydroxychloroquine, azathioprine, cyclosporine and, in life-threatening cases, cyclophosphamide and corticosteroids. Isolated reports have suggested the benefits of using an oral cyclosporine in pSS patients with interstitial cystitis. In patients with active pSS, rituximab, belimumab and abatacept have also been used, but this treatment is uncommon due to its high cost, and questions relating to its usefulness in pSS still remain unanswered.[21–24]

In conclusion, pulmonary involvement in pSS is common and occurs in around 30% of patients. This represents one of the most intriguing aspects of the disease. The main symptom is persistent dry cough that periodically wakes up patients at night. Radiological tests in daily clinical practice allow diagnosis of pulmonary findings, even if the changes are not very advanced. Additionally, they enable monitoring of pSS activity, because a factor or factors predisposing to the development of changes in the respiratory system have not been found. Changes in HRCT occur more frequently only in patients with peripheral arthritis.

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Continent catheterizable conduits in pediatric urology: One-center experience

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Abstract

Background. Clean intermittent catheterization (CIC) is a standard treatment for patients who are unable to empty the bladder. In the absence of the urethra or if catheterization through the urethra is problematic, a continent vesicostomy is used as a catheterizable conduit. The Malone procedure is an established treatment option for children with neurogenic constipation and fecal incontinence.

Objectives. The aim of the study was to report the authors' experience with continent catheterizable conduits (CCCs) in children, to review the results and to determine the efficacy of the technique, with an emphasis on continence and the need for revision.

Material and methods. The retrospective study involved children who underwent catheterizable conduit procedures from 2000 to 2015. Two kinds of continent stomas were performed: Mitrofanoff vesicostomies for CIC and Malone antegrade continence enemas (MACEs). The 115 patients treated included 66 girls and 49 boys. A total of 134 operations were performed; 62 were Mitrofanoff vesicostomies and 72 were Malone appendicostomies. In 19 cases, both Mitrofanoff and Malone appendicostomies were formed out of 1 appendix divided into 2 parts. In 5 children vesicocutaneous stomas were constructed using Monti's procedure, and in 1 it was constructed from an intussuscepted ileal loop. In 27 patients Malone procedures were performed laparoscopically.

Results. The mean follow-up period was 8.6 years. There was no serious morbidity in relation to the surgery. In 9 children local wound infection was noted, and in 9 others stomal stenosis developed. Out of the 62 children with catheterizable vesicostomies, 59 were continent. The MACE procedure was successful in all 72 patients; problems with constipation and fecal incontinence were resolved in all cases. None of the laparoscopies needed conversion.

Conclusions. Continent catheterizable conduits help patients achieve both fecal and urinary continence. Laparoscopy is effective in performing the Malone procedure. Stoma-related complications could be avoided using end-to-side appendix anastomoses to the skin. Stomal incontinence is rare even when a simplified technique is employed, using the appendix without cecoplication.

Key words: surgery, urinary incontinence, child, neurogenic bladder, fecal incontinence

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Severe impairment of bladder function can arise from various causes. The most common cause of bladder dysfunction in children is spinal dysraphism. Other causes, such as developmental defects (an imperforate anus, sacral tumors or sacral agenesis), accidental injuries or spinal tumors are rare.[1–3]

In children with severe bladder dysfunction, the therapeutic strategy is focused on 2 tasks: preservation of renal function and control of micturition, maintaining urinary continence. For those purposes, a continent, low-pressure, high-volume bladder is essential.

This is achieved by ensuring complete emptying of the bladder.[1–3]

Since 1972, clean intermittent catheterization (CIC) has been the standard treatment for patients who are unable to empty the bladder.[4] If catheterization through the urethra is problematic or in rare cases of the absence of the urethra, a continent vesicostomy (CVS) is performed to create a continent catheterizable conduit (CCC). In Paul Mitrofanoff's original description from 1980, the CCC is made from the appendix.[5–7]

With improved urological care and the introduction of CIC and oral anticholinergics, the majority of children with neurogenic bladders survive into adulthood expecting the best quality of life. Decreased bladder capacity can be effectively treated with oral anticholinergics, but in some children, bladder augmentation or bladder replacement has to be performed. CIC is mandatory to empty an augmented bladder; in some children the use of a Mitrofanoff stoma is required.[5–8]

Children with neurogenic bladder also encounter serious problems with defecation and fecal incontinence. The first line therapy for neurogenic constipation is conservative treatment with dietary recommendations, mineral-based and osmotic sugar laxatives, suppositories and enemas. In most cases, this kind of treatment proves very effective. Children with meningomyelocele are especially difficult to deal with because constipation caused by prolonged colonic transit time is accompanied by fecal and gas incontinence caused by sphincter dysfunction. Surgical treatment is considered in patients for whom non-invasive treatment methods related to stool consistency, its retention and the ability to control defecation have proved ineffective. The Malone antegrade continence enema (MACE) procedure is an established treatment option for children with chronic neurogenic constipation and fecal incontinence. The aim of the Malone procedure is to use the appendix as a CCCs for antegrade colonic enema (ACE).[9–12]

The aim of the study was to report the authors' experience with CCCs in children, to review the results and to determine the efficacy of the technique, with an emphasis on continence and the need for revision.

Table 1. The types of conduit procedures performed

Procedures	Number of children
Creation of CVS into the native bladder including:	11
• using the appendix	6
• using the Monti technique	3
Creation of CVS for BA or BR including:	51
• using the appendix	48
• 2 stomas (MACE + CVS) from a divided appendix	19
• using the Monti technique	2
• CVS from intussuscepted ileal loop	1
MACE appendicostomy (open surgery) including:	45
• 2 stomas (MACE + CVS) from a divided appendix	19
Laparoscopic assisted MACE appendicostomy	27

CVS – continent vesicostomy; BA – bladder augmentation; BR – bladder replacement; MACE – Malone antegrade continence enema.

Material and methods

The retrospective study included children who underwent CCC procedures at the Department of Pediatric Surgery at Poznan University of Medical Sciences (Poland) between 2000 and 2015. Two kinds of continent stomas were performed: Mitrofanoff CVS for CICs and Malone appendicostomies for ACE procedures. A total of 115 patients were treated, comprising 66 girls and 49 boys. The patients' mean age at the time of the operation was 9.4 years (age range: 2–17 years). A total of 134 CCCs were created by a single surgeon, of which 62 (46%) were CVS for CICs and 72 (56%) were MACE appendicostomies; in 19 cases (14%) both stomas were created from a single divided appendix. The types of conduit procedures are shown in Table 1.

Continent vesicostomies

In the study period, 62 CVSs were performed to create channels for CICs. The mean age of the children who underwent this procedure was 8.8 years (age range: 2–17 years). The Mitrofanoff operation was performed to provide alternative access to the bladder. It was performed as an additional procedure accompanying bladder augmentation, or as a separate operation in boys with preserved sensation in the urethra (in the case of boys who had undergone bladder exstrophy and boys with complications following posterior urethral valve resection).

CVS was also proposed for girls with neurogenic bladder who had technical problems with self-catheterization and for all patients undergoing bladder replacement procedures. In 46 children, CVS was an additional procedure at the time of bladder augmentation (BA); in 5 it was performed during a bladder replacement (BR) operation; in 11 children CVSs were created for CIC of a native neurogenic bladder; and in 19 cases both Mitrofanoff and

Malone appendicostomas were formed out of 1 appendix divided into 2 parts.

The appendix was used for CVS whenever possible; in 5 cases a flap of the small intestine was employed using the Monti technique, and in 1 case a catheterizable stoma was made of an intussusception ileal nipple.

MACE procedures

The 64 patients qualified for MACE operations were those with chronic intractable neurogenic constipation and fecal and gas incontinence. The mean age of these patients was 9.3 years (age range: 5–17 years). In 54 of these children defecation problems were caused by a dysraphic defect of the lumbar and sacral regions of the spine; in 3 patients the problems were caused by sacral agenesis; and in 1 patient by a sacrococcygeal tumor. In 4 of the children, constipation and fecal incontinence remained after a posterior sagittal anorectoplasty for an imperforate anus. In all the MACE operations, the appendix was used as a CCC.

The underlying causes of the need for a CCC are listed in Table 2.

Table 2. The causes of bladder and bowel dysfunction

Cause of dysfunction	Number of children	
	MACE	CVS
Status post cystectomy rhabdomyosarcoma (RMS)	-	5
Open meningomyelocele (MMC)	56	32
Other defects of the nervous system (occult spinal dysraphism, sacral agenesis, tumor)	4	7
Cerebral palsy	-	3
Bladder exstrophy	-	7
Posterior urethral valves	-	6
Imperforate anus	4	2
Total	**64**	**62**

The MACE operations were carried out either by classic open surgery or by a laparoscopically assisted technique (LACE procedure). The classic operations were performed on 45 patients as an additional procedure during the augmentation of the bladder. In the 27 patients who underwent the MACE operations as a separate procedure, it was performed laparoscopically.

In the LACE operations, an umbilical port was used for the camera with 2 or 3 additional ports. Two ports were used in 25 patients, and in the 2 remaining cases, a 3rd port was used. Once the cecum and appendix were located, mobilized and freed of any adhesions, a skin incision was made and the distal part of the appendix was brought out of the peritoneal cavity with the aid of laparoscopic tools. In the first 23 procedures, an anastomosis of the spatulated tip of the appendix with a tubularized skin flap was performed.

In subsequent operations, a simplified procedure was used: the distal tip of the appendix was anastomosed directly end-to-side to the skin tube. An 8Fr Foley catheter was used to stent the appendicocutaneous anastomosis for 2 weeks.

The original description of the Malone procedure relied on a reversed, tunneled and reimplanted appendix. In all the cases in the present study, a simplified in situ procedure was performed, using the appendix as a CCC without cecoplication. This requires minimal mobilization of the appendix and minimal manipulation of the blood supply. In neither Mitrofanoff nor Malone procedures there was any anti-reflux tunneling of the proximal part of the appendix performed, as the continence mechanism is a function of the appendix length and the mucosal coaptation of the appendiceal lumen. Especially in cases in which MACE and CVS were formed from a divided appendix, the length of the 2 conduits was too short to allow a surgical creation of any rational anti-reflux mechanism.

Starting 48 h after the operation, daily infusions were made through a catheter.

The catheter remained in the stoma for at least 2 weeks after the operation. Afterwards, patients catheterized their MACE stoma channels every day, making infusions every 2nd or 3rd day.

Results

The mean follow-up period was 8.6 years (ranging from 1 to 15 years). There was no mortality or serious morbidity in relation to the surgery. In 7 children (9.7%) who underwent MACE procedures and in 2 (3.2%) who underwent CVS, local wound infection was managed conservatively; however, partial dehiscence in the skin part of the fistula in 2 children resulted in channel shortening. In 1 patient, complete destruction of the skin part of the channel occurred.

All MACE and CVS stomas were catheterized easily with a 6–10 Fr feeding tube. Stomal stenosis requiring dilation in the office was observed in 6 children (8.3%) who had had MACE procedures and in 3 (4.8%) of those who had had CVS procedures. Four children were reoperated. All 9 of these strictures developed in the first 6 months after the surgery, and they were all observed in the cases of cutaneo-appendico anastomosis with a tubular skin flap.

Out of the 62 CVS children, 59 (95%) were continent. One child with an appendix CVS and 1 with a Monti CVS had mild urine leakage at maximal bladder capacity; and a girl who had had BR surgery and a CCC made from an intussusception ileal nipple remained incontinent after reoperation and finally decided to be diverted with a Bricker stoma.

In all the children in the study, the MACE operation was successfully performed by both classic and LACE

techniques. There was no need for conversion in any of the LACE procedures. The laparoscopic method permitted precise location of the appendix and its mobilization, freeing it from any adhesions, and made it easy to bring the appendix out of the peritoneum. The use of a 3rd port for the needle-holder to fix the cecum to the undersurface of the abdominal wall prolonged the procedure and made it more complicated.

Following MACE procedures, 59 (92%) out of 64 patients were continent; in 5 patients (7.8%) mucus leakage from the MACE stoma was observed and treated with dressing. One patient underwent successful surgical revision of a stoma with cecoplication.

In long term observation, 92% of the MACE conduits were still in use. In 3 children (4.6%), the MACE stoma closed because of discontinued catheterization. In 1 child, the perforation of the MACE channel was treated conservatively, but the stoma closed.

Problems with constipation and fecal incontinence were resolved in all cases of the MACE operation. In 1 patient, voluntary defecation without the need for infusions was observed within a few months after the ACE treatment. The patient's parents decided to give up catheterization and ACE.

The overall complications rate is shown in Table 3.

Table 3. Complications of CCC procedures

Complication	Number of children	
	MACE	CVS
Wound infection	7	2
Stoma stenosis	6	3
Leakage of urine	–	3
Mucus leakage	5	–
Stoma closure	5	–
Stoma perforation	1	–
Reoperation	3	3
Total	27 (42%)	11 (17%)

Discussion

The aim of conduit procedures is to provide a channel for intermittent catheterization that is continent, easily accessible and painless. The indications for constructing a CCC are neurogenic bladder, inability to void with urine incontinence and refractory neurogenic constipation with fecal incontinence.

Great improvements in the treatment of patients with neurogenic bladder was made with the introduction of CIC by Jack Lapides in 1971. The next important step was made by Paul Mitrofanoff in 1980, who proposed using the appendix as an alternative continent channel for CIC in patients with urethral strictures. Other tubular structures have also been proposed as CCC alternatives when the appendix is not available, including a fallopian tube, the ureter or a short segment of retubularized small bowel (the Monti technique).[5–7,11–17]

CIC with a CCC enabled further development of reconstructive urology by applying the ideas of Jan Mikulicz-Radecki, a surgeon based in Wrocław, Poland, who in 1899 was the first to describe the use of parts of the digestive tract for bladder reconstruction.[18]

The mean age of the children in the present study was 9.4 years, similar to those in the studies by VanderBrink et al., Bani-Hani et al. and Süzer et al.[5,12,13] In the majority of cases the bladder and bowel dysfunctions were caused by meningomyelocele. Those observations are consistent with other studies.[6,12–14]

In the present study, CCCs for bladder CIC were created from the appendix in the majority of the patients (128), in 19 children they were formed from a divided appendix, and 5 Monti tubes were constructed. Similar ratios were reported by Süzer and Castellan.[5,16]

In augmented patients, CVS was proposed as an additional procedure, especially for boys with preserved urethra sensation and for girls with technical problems with self-catheterization. In children selected for the Mitrofanoff procedure on their native bladders, no bladder neck surgery was performed, as all of them had proper age-related bladder volume with leak-point pressure >20 cm H_2O.

Most of the children in the present study became continent: 95% of those who underwent CVA and 92% of those who had MACE procedures. One girl with a CVS made of an intussuscepted ileal loop remained completely incontinent, and was diverted. In 1 boy with an appendiceal CCC and 1 with a Monti tube, minor leakage from the CVS was observed at maximal bladder capacity, with no need for correction in the patients' opinion. The continence rate in the patients in the present study is similar to that reported by Clark et al., Farrugia and Malone, and Castellan et al., with similar complication rates.[14–16]

McAndrew and Malone assessed the outcomes of 112 CCC channels, both CVA and MACE, and did not find any difference in the incidence of complications between the 2 types of conduit. They reported that 93% of the MACE conduits were continent, but stomal stenosis occurred in 29% of the CVS; stenosis was less common with Monti tubes than with appendix CCCs.[17] Castellan et al. found no difference in the incidence of complications in Monti vs appendix conduits.[16]

Chronic constipation accompanied by fecal and gas incontinence is a real problem in patients suffering from a neurogenic bladder. The mechanisms leading to neurogenic constipation and urine incontinence are similar. Spine malformations or injuries in the lumbosacral region can damage somatic and autonomic sensory and motor fibers, as well as spinal centers. All the patients in the present study had unsuccessfully tried conservative treatments, following dietary recommendations, oral herbal and synthetic, osmotic and stimulant laxatives

and, as a last resort, used suppositories and colonic enemas. Whenever that kind of treatment proved ineffective, surgery was recommended.

The idea of using the appendix as a CCC in children with chronic constipation was conceived in 1989 by P.S. Malone. This stoma was supposed to serve for isoperistaltic antegrade colonic enema (ACE) for colonic washout. A year later, the author described this method in *The Lancet*. In the first 21-patient group assessed by the author, the continent appendicocecostomy was created with or without reversal of the appendix. The tip of the appendix was anastomosed to a skin tube to avoid problems with exposed mucosa.[9–11,14] Many modifications of the original technique have been described. This stoma has been applied in both the ascending and descending colon.[19] The Malone operation can be an additional procedure during the augmentation and reconstruction of the bladder.[11,14–16] When the surgical anatomy is amenable, the appendix can be used for the creation of both MACE and CVA conduits. The split-appendix technique was used in 14% of the patients in the present study. In a series of 394 children reported by VanderBrink et al., the split-appendix technique was used in 11% of the patients.[12]

The MACE operation was originally described by Malone as a procedure performed in the classic fashion with 2 incisions. This has been adapted to a laparoscope-assisted technique (LACE). To perform a LACE procedure 2 ports are needed, and an incision in the skin made in a place chosen during the laparoscopy, which is then used to make the stoma outlet. In the LACE technique, the appendix is not reversed.[19–22]

In 2 patients in the present study, a 3rd port was used for the needle-holder for the purpose of fixing the cecum to the abdominal wall. This solution, however, was not useful. Manipulation with the needle-holder is complicated and makes the procedure longer in comparison to the simplified technique, where the cecum is left untouched.

The LACE operation is not always possible and some patients need conversion to classic operation. However, in the present study none of the LACE patients required conversion.

In the first 18 patients, the end portion of the MACE stoma was made of a flap of skin and joined to the end of the appendix. Creating such a skin channel results in a better cosmetic effect, although it may cause complications.[11,12,14,16,17,20] In 3 of the LACE patients in the current study, local infection of the wound occurred, and in 6 of those patients strictures developed. According to other authors, such minor complications may be present in 10–81% of MACE operations, with stoma stenosis in about 20–30%. The results of the present study are comparable with those from the literature, but with a lower incidence of stomal stenosis.[12–14,16,17,23,24]

Narayanaswamy et al. suggested that 26% of patients with appendicovesicostomy and 60% of patients with ileovesicostomy have difficulty with catheterization.[24] To avoid complications of stenosis, the current authors suggest not reversing the appendix for CVS and MACE. It is preferable not to do any cecoplication with direct anastomosis of the conduit to the skin.

Another complication after Malone procedures is mucus leakage from the stoma, occurring in 5-15% of patients.[10–12,14,16,17,23,24] In the present study, it was seen in 7.8% of children.

In long-term observation, 92% of the conduits in the present study group were still in use, which is similar to what was reported by Farrugia and Malone, and Lamelle et al.[15,25]

Although no procedures aimed at creating valve mechanisms between the appendix and the cecum were performed in the present study, the number of patients with complications was close to that reported in the literature.[10–12,14,16,17,23–25] Secondary ischemia, adhesions and scar formation are reduced, alleviating the most common complication, stoma stenosis. These results also show that cecoplication is not necessary to maintain stomal continence after MACE.

Conclusions

Continent catheterizable conduits help patients achieve both fecal and urinary continence.

Laparoscopy is effective in performing Malone operations.

Stoma-related complications can be avoided using end-to-side appendix anastomoses to the skin. Stomal incontinence is also rare when a simplified technique, using the appendix without cecoplication, is employed.

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Cerebral venous thrombosis as a diagnostic challenge: Clinical and radiological correlation based on the retrospective analysis of own cases

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Abstract

Background. Cerebral venous thrombosis (CVT) is a rare condition which constitutes 0.5–1% of all strokes. The clinical and radiological picture of CVT is non-specific and can mimic other disorders.

Objectives. The aim of the study was to retrospectively evaluate and correlate clinical and radiological symptoms presented by patients with CVT, both in the initial and follow-up neurological and neuroimaging examinations, with a special emphasis on diagnostic difficulties.

Material and methods. Material consisted of 11 patients with CVT (7 women, 4 men). The average age was 43.5, ranging from 23 to 69 years. Clinical symptoms, laboratory findings, risk factors and the results of neuroimaging examinations including CT, MRI and DSA were retrospectively analyzed and correlated.

Results. All subjects developed superficial CVT and 1 also deep CVT, with no parenchymal lesions in 2 cases, non-hemorrhagic infarctions in 3 and hemorrhagic lesions in 6 subjects. The most frequent symptoms were headache, seizures and hemiparesis. The major risk factors were hormonal therapies in women and congenital thrombophilia. Factors influencing the clinical course and outcome the most were location and type of brain lesions, with hemorrhagic cortical infarctions bringing the worst prognosis and being associated with the highest rate of persistent neurological deficits, despite the rate of vessel recanalization.

Conclusions. In our opinion, quick diagnosis before parenchymal hemorrhagic lesions are visible on CT is of crucial importance and requires a constant alertness and good cooperation of neurologists and radiologists, especially in emergency settings.

Key words: cerebral venous thrombosis, headache, diagnostic imaging

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Cerebral venous thrombosis (CVT) is a rare condition which constitutes 0.5–1% of all strokes and may involve both intracranial veins and sinuses.[1,2] The clinical picture of CVT is non-specific and can mimic other neurological disorders. The most common neurological symptoms include headache or features of increased intracranial pressure as well as seizures and focal neurological deficits, which depend on the location of cerebral lesions.[3] Involvement of the superior sagittal sinus usually leads to motor deficits and seizures while thrombosis of the left transverse sinus may cause aphasia. Rare symptoms include cavernous sinus syndrome (exophthalmos, conjunctival edema and painful ophthalmoplegia), tinnitus, isolated psychiatric symptoms and cranial nerve palsies.[4]

The etiology of CVT is very similar to that of deep vein thrombosis in the legs and can be divided into non-infectious and infectious factors. The non-infectious factors are oral contraceptives, pregnancy and puerperium, hormonal disorders, severe dehydration, cancer, connective tissue disease, trauma and neurosurgical procedures as well as hematological disorders such as deficiency of protein C, S or antithrombin III, mutations of factor V Leyden or prothrombin genes and essential thrombocythemia or polycythemia. The tendency to thrombosis also occurs in antiphospholipid syndrome, circulatory disorders, and in the course of prolonged immobilization of the patient. Central nervous system or ear infections, sinusitis, endocarditis and sepsis are the most common infectious risk factors of CVT.[1,5,6] In 44% of CVT patients, more than 1 risk factor may be found while in 15% the etiology of CVT remains unknown.[7]

Due to non-specific clinical symptoms, neuroimaging examinations play a major role in the initial diagnosis of CVT. Since CVT patients usually develop acute neurological symptoms, the first imaging examination is CT of the brain in the emergency department. CVT affects cerebral veins and dural sinuses, and perhaps leads to lesions within brain parenchyma such as edema or venous infarction. To visualize the whole extent of the disease, an initial CT examination of the brain is often followed by other imaging examinations such as CT venography, magnetic resonance (MR) with or without contrast injection, MR venography (MRV) and, very rarely, digital subtracted angiography (DSA).[8,9] Accurate diagnosis of CVT allows for quick implementation of treatment and better prognosis for the patient.[10]

It has to be stressed that despite the growing knowledge of this disease, the diagnosis of CVT is still commonly overlooked and delayed due to the remarkable diversity of clinical symptoms and neuroimaging appearance, and thus still remains a diagnostic challenge for both clinicians and radiologists.

The aim of the study was to retrospectively evaluate and correlate clinical and radiological symptoms presented by patients with CVT both in the initial and follow-up neurological and neuroimaging examinations, with a special emphasis on diagnostic difficulties.

Material and methods

Eleven patients with CVT (7 women, 4 men) hospitalized in the Department of Neurology at Wroclaw Medical University between 2009 and 2014 were involved in the retrospective study. The average age was 43.5, ranging from 23 to 69 years. All patients underwent detailed neurological evaluation by experienced neurologists as well as laboratory examinations including the assessment of the coagulation parameters.

All patients underwent several neuroimaging examinations, including CT and MR examinations in all subjects and DSA in 2 cases. All CT examinations were performed using a 64 row scanner (GE Healthcare) with a slice thickness of 0.625 mm, and included unenhanced and contrast enhanced CT examinations as well as CT venography. All MR examinations were performed using a 1.5 T MR scanner (Signa Hdx GE). The analyzed MR examinations included standard MR examinations (T1-, T2-weighted images, FLAIR sequence, diffusion weighted imaging – DWI, susceptibility weighted imaging – SWI), without or with contrast administration, as well as MR venography (MRV) using a 3D Time of Flight (TOF) sequence. The study was conducted in accordance with the guidelines of the University Ethics Committee for conducting research involving humans. Each patient provided signed informed consent to participate in the examination.

Clinical symptoms, laboratory findings, risk factors and the results of neuroimaging examinations were retrospectively analyzed. Neurological examinations and neuroimaging examinations were performed on admission and then several times during the follow-up period. The follow-up period ranged from 2 weeks to 45 months (mean 39 weeks). A constant monitoring after hospitalization covered 9 patients with CVT, with the observation time ranging from 8 weeks to 45 months. Two patients did not present themselves for follow-up examinations after hospitalization.

Results

Clinical symptoms

The most common clinical symptoms prior to hospitalization were severe headache (8 patients), hemiparesis (7 patients) and loss of consciousness with seizures (6 patients). In 2 subjects headache was the only symptom, in the rest headache was accompanied by other symptoms such as epilepsy (2 subjects), hemiparesis (2 subjects) or both epilepsy and hemiparesis (2 subjects). Three patients showed aphasic speech disorders, and 1 patient experienced visual phenomena and transient prosopagnosia (Tables 1–3).

Follow-up examinations revealed overall improvement in the neurological status of all patients. Four patients fully recovered and showed no neurological symptoms and deficits, 2 patients developed persistent hemiparesis,

3 patients secondary epilepsy and 2 subjects both hemiparesis and secondary epilepsy (Tables 1–3).

Laboratory findings

Laboratory tests carried out on admission revealed slightly elevated levels of C-reactive protein (CRP) and leukocytes in 3 patients, elevated levels of D-dimers (2.12–22.06 μL/mL) in 5 subjects and elevated levels of fibrinogen (4.7–7.5 g/L) in 4 patients. Only 3 of 11 patients revealed abnormalities in coagulation status such as resistance to activated protein C, protein S deficiency and prothrombin G2021A mutation. Three patients underwent CSF examination which showed elevated protein levels in 2 subjects.

Risk factors

Risk factors of CVT were established in 8 patients while in 3 cases no risk factors were found. The most frequent risk factors were hormonal therapies (oral contracep-

Table 1. Neurological and radiological findings in patients with no brain lesions due to cerebral venous thrombosis

Patient No.	Age, sex	Location of vascular and parenchymal changes based on neuroimaging studies	Neurological status on admission and before	Risk factors	Follow-up neuroimaging studies	Neurological status in the follow-up examinations
1.	40 years, female	vessels: right IJV, transverse and sigmoid sinuses with infratentorial cortical veins and posterior aspect of superior sagittal sinus with left draining cortical veins brain: no lesions	admission: severe headache, normal neurological examination before: severe headache lasting for a few days	hormonal infertility treatment, prothrombin G20210A mutation	after 9 weeks (MR + C + MRV) vessels: partial recanalization	after 9 weeks normal neurological examination, no neurological symptoms
2.	23 years, male	vessels: right transverse and sigmoid sinuses, IJV brain: no lesions due to cerebral venous thrombosis	admission: severe headache, peripheral damage of the 7th nerve on the right, right-sided hearing loss due to right temporal bone fracture before: severe head injury 5 days earlier	fracture of the right temporal bone close to the right sigmoid sinus	after 9 weeks (MR + C + MRV) vessels: total recanalization	after 9 weeks normal neurological examination, no neurological symptoms

IJV – internal jugular vein; MR + C + MRV – MR examination with MR venography and contrast administration.

Table 2. Neurological and radiological findings in patients with non-hemorrhagic brain lesions due to cerebral venous thrombosis

Patient No.	Age, sex	Location of vascular and parenchymal changes based on neuroimaging studies	Neurological status on admission and before	Risk factors	Follow-up neuroimaging studies	Neurological status in the follow-up examinations
3.	42 years, female	vessels: left vein of Labbe, transverse and sigmoid sinuses, IJV brain: non-hemorrhagic edema within the cortex of the left temporo-parieto-occipital region	admission: sudden speech impairment, mixed aphasia, moderate right-sided hemiparesis before: no symptoms	not found	after 2 weeks (MR + C + MRV) vessels: no recanalization brain: non-hemorrhagic edema in the left temporo-parieto-occipital region	after 2 weeks mild right-sided hemiparesis, no further follow-up available
4.	40 years, female	vessels: anterior aspect of the superior sagittal sinus and left draining cortical veins brain: non-hemorrhagic edema within the cortex of the left frontal lobe	admission: generalized seizures with tongue biting, normal neurological examination before: severe headache and behavioral changes for a few days	oral contraception, protein S deficiency	after 9 weeks (MR + C + MRV) vessels: total recanalization brain: no visible lesions	after 9 weeks symptomatic epilepsy, normal neurological examination
5.	31 years, female	vessels: left vein of Labbe, transverse and sigmoid sinuses, IJV deep venous system: straight sinus, vein of Galen, internal veins of the 3rd ventricle, inferior sagittal sinus brain: non-hemorrhagic edema of the left thalamus, caudate nucleus and left periventricular white matter	admission: sudden transient loss of consciousness, mild mixed aphasia and mild right hand hemiparesis with positive right Babinski sign before: severe headache of the left temporo-occipital region with nausea and vomiting for a week	oral contraception	after 8.5 months (MR + C + MRV) vessels: total recanalization brain: small T2-hyperintense foci within the left periventricular white matter	after 8.5 months normal neurological examination, no neurological symptoms

IJV – internal jugular vein; MR + C + MRV – MR examination with MR venography and contrast administration.

Table 3. Neurological and radiological findings in patients with hemorrhagic brain lesions due to cerebral venous thrombosis

Patient No.	Age, sex	Location of vascular and parenchymal changes based on neuroimaging studies	Neurological status on admission and before	Risk factors	Follow-up neuroimaging studies	Neurological status in the follow-up examinations
6.	29 years, female	vessels: superior sagittal sinus with draining cortical veins, bilateral transverse and sigmoid sinuses, bilateral IJVs brain: hemorrhagic infarction in the right frontal lobe	admission: transient loss of consciousness two times, generalized seizures with convulsions, mild left-sided hemiparesis before: severe headache for a few days	caesarean section 10 days earlier, resistance to the activated protein C	after 3.5 months (MR + C + MRV) vessels: total recanalization brain: postinfarction malacia within the cortex of the right frontal lobe	after 3.5 months symptomatic epilepsy, mild mouth asymmetry on the left side
7.	49 years, male	vessels: superior sagittal sinus, bilateral transverse and sigmoid sinuses, straight sinus, right vein of Trolard brain: 2 bilateral fronto-parietal hemorrhagic infarctions	admission: complex partial seizures with moderate left side hemiparesis, psychomotor slowing before: severe headache	malignant melanoma with distant metastases	after 23 days (CT) vessels: unknown status brain: blood resolution within infarctions	after 23 days motor aphasia, mild right-sided hemiparesis
8.	36 years, female	vessels: right vein of Labbe, transverse and sigmoid sinuses, IJV brain: right fronto-temporal cortical hemorrhagic infarction	admission: generalized seizures, mouth asymmetry on the right side, delays in the left upper limb in the Barre attempt before: severe headache of the occipital region for a few days,	oral contraception	after 34 months (CT + CTA) vessels: total recanalization brain: postinfarction malacia in the right temporal cortex	after 34 months symptomatic epilepsy, normal neurological examination
9.	59 years, female	vessels: right superior sagittal sinus, transverse and sigmoid sinus, IJV brain: left fronto-temporal cortical hemorrhagic infarction with large mass effect and regional SAH	admission: focal seizures, conscious, deep sensorimotor aphasia, moderate right-sided hemiparesis with a positive right Babinski sign before: mixed aphasia and mild right-sided hemiparesis for 2 days	colon cancer treated with radio and chemotherapy 2 years earlier no other risk factors found	after 45 months (MR + C + MRV) vessels: partial recanalization brain: post-infarction malacia in the left temporal cortex	after 45 months symptomatic epilepsy, sensorimotor aphasia, mild right-sided hemiparesis
10.	69 years, male	vessels: right transverse and sigmoid sinuses, IJV brain: right occipital cortical/subcortical hemorrhagic infarction	admission: visual phenomena, bradyphrenia, transient prosopagnosia, mild left-sided hemiparesis before: headache for 2 weeks	not found	after 8 months (MR + C + MRV) vessels: partial recanalization brain: post-infarction malacia within the right occipital cortex	after 8 months normal neurological examination, no neurological symptoms
11.	61 years, male	vessels: anterior aspect of the superior sagittal sinus brain: left frontal hemorrhagic infarction	admission: increasing memory and orientation impairment, psychomotor slowing, mild right-side hemiparesis before: first generalized seizures 2 months earlier	not found	after 8 months (MR + C + MRV) vessels: no recanalization brain: post-infarction malacia in the cortex of the left frontal lobe	after 8 months symptomatic epilepsy, mild right-sided hemiparesis

IJV – internal jugular vein; MR + C + MRV – MR examination with MR venography and contrast administration.

tives, infertility treatment) found in 4 cases, followed by abnormalities in the coagulation process in 3 cases, neoplasmatic process in 2 patients, a fracture of the temporal bone in 1 patient and a cesarean section in 1 case. In 3 cases of female patients, several risk factors were present such as a cesarean section and resistance to activated protein C, hormonal infertility treatment coexisting with prothrombin G20210A mutation, or oral contraception with a protein S deficiency (Tables 1–3).

Treatment

Immediately after admission, all patients received symptomatic treatment depending on the patient's condition and symptoms, i.e., treatment of intracranial hypertension, seizures or headache. After the final diagnosis of CVT, according to the recommendations of the European Federation of Neurological Societies, all patients received body-weight adjusted subcutaneous low-molecular-weight

Table 4. Initial diagnosis and a list of neuroimaging studies performed in patients with cerebral venous thrombosis during hospitalization. A neuroimaging examination which enabled a correct diagnosis is indicated in bold

Patient number	First radiological diagnosis based on an emergency CT	Neuroimaging studies during hospitalisation
1.	cerebral venous thrombosis	CT (1st day), CTV, MR + C + MRV
2.	temporal bone fracture, artefacts	CT, CT (2nd day), CTV, MR + C
3.	cerebral venous thrombosis	CT (1st day), CTV, MR + C + MRV
4.	normal	CT, MR + C (6th day)
5.	normal	CT, CT, CT + C (5th day), CTV, MR + C + MRV, CT, CTV
6.	bleeding vascular malformation	CT, DSA, MR + C (5th day), CT + C
7.	hemorrhagic metastases	CT + C, CT, CT + C (8th day), MR, CT
8.	normal	CT, CTV, MR + C (8th day), MR, CT
9.	unequivocal diagnosis: hemorrhagic ischemic or venous infarction or brain contusion	CT + C, CTV (3rd day), CT, CT
10.	hemorrhagic brain contusion	CT, CT + C, MR + C (13th day), MR, MR + C
11.	unequivocal: hemorrhagic brain contusion or metastases	CT, MR + C, CTV, DSA (21st day)

CT – unenhanced computed tomography; CT + C – contrast enhanced computed tomography; CTV – computed tomography venography; MR – unenhanced magnetic resonanse; MR + C – contrast enhanced magnetic resonanse; MRV – magnetic resonance venography; DSA – digital subtracted angiography.

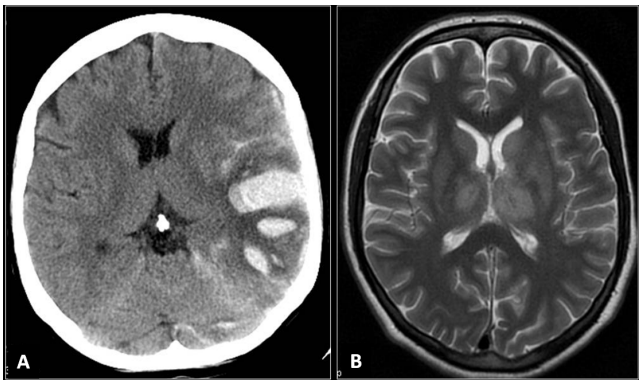
heparin at therapeutic doses and transitioned to vitamin K antagonist.[11] After discharge, all patients were on oral anticoagulants based on a moderate international normalized target ratio (INR) between 2.0 and 3.0. The duration of the treatment depended on the risk factors. All patients with provoked CVT, associated with transient risk factors such as head trauma and oral contraceptives, were treated with a vitamin K antagonist for 3 months. Patients with unprovoked CVT received therapeutic anticoagulation for 6–12 months. Patients with thrombophilia were treated for 6–12 months and in these cases indefinite anticoagulation therapy should be considered. In 5 cases, additional antiepileptic drugs were included in the constant therapy because of the diagnosis of secondary epilepsy.

Neuroimaging examinations

All patients underwent an emergency CT scan of the brain, in 9 cases without intravenous contrast injection and in 2 cases with contrast administration. On the basis of the emergency brain CT, only 2 patients were correctly diagnosed with CVT. In the remaining patients, other diagnoses were suggested such as bleeding vascular malformation (1 subject), hemorrhagic metastases (1 subject) or hemorrhagic cerebral contusion (1 subject). In 2 patients, the CT images were reported as inconclusive requiring differentiation between hemorrhagic arterial or venous infarction, cerebral contusion or hemorrhagic metastasis. In 1 patient after a severe head trauma, signs of CVT were overlooked due to motion artefacts. In 3 patients, the CT examinations were reported as normal (Table 4).

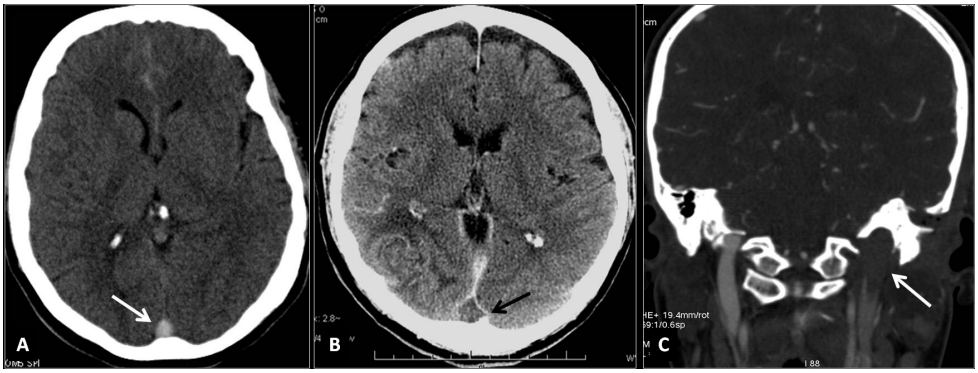
During hospitalization, all patients underwent several neuroimaging examinations such as CT with or without contrast administration, CT venography, MRI with or without contrast administration, MR venography (MRV) and digital subtracted angiography (DSA) (Table 4, Fig. 1–3). In 9 patients, a correct diagnosis of CVT was established in the follow-up examinations, such as unenhanced CT (in 1 subject on the 2nd day), contrast enhanced CT (in 2 subjects on the 5th and 8th day), CT venography (in 1 subject on the 3rd day), contrast enhanced MRI (in 4 subjects on the 5th, 6th, 8th and 13th day), and in DSA (in 1 subject on the 21st day). Diagnosis of CVT took an average of 6.6 days (from 1 to 21 days). Only in 2 patients was CVT diagnosed during the first neuroimaging examination, the rest of the

Fig. 1. Brain lesions in the course of CVT



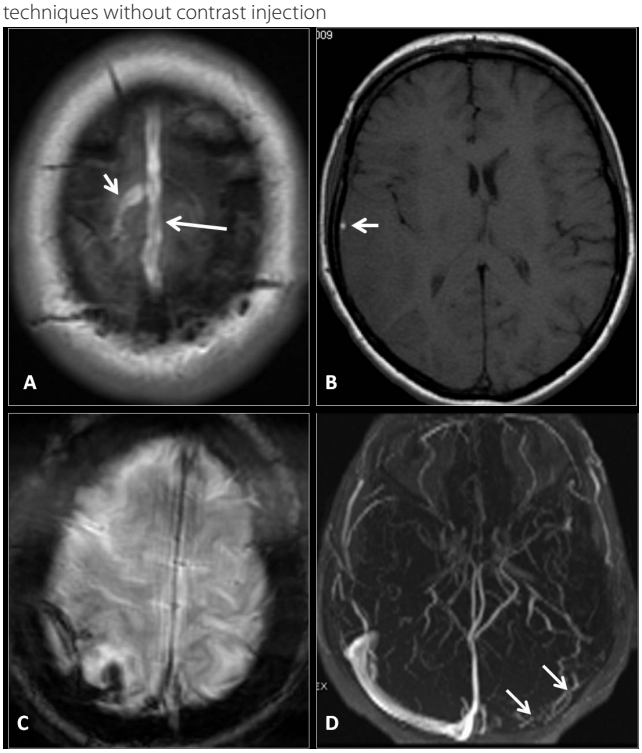
a – a CT image showing typical hemorrhagic venous infarction in the left temporal lobe; b – T2-weighted MR image showing non-hemorrhagic edema within the left basal ganglia region and both thalami due to thrombosis of the deep venous system, which was not visible in the follow-up MR examinations.

Fig. 2. Imaging of thrombosed vessels with CT techniques



a, b – thrombosis of the posterior aspect of the superior sagittal sinus. The thrombosed sinus is visible as a hyperdense vessel (arrow) on a non-enhanced CT image (a) or as a filling defect (arrow) on the contrast enhanced CT image (b); c – a coronal CT angiography image showing a large filling defect within a thrombosed left internal jugular vein (arrow).

Fig. 3. Imaging of thrombosed sinuses and cortical veins with MR techniques without contrast injection



a, b – T1-weighted images showing hyperintense thrombosed superior sagittal sinus (large arrow) and cortical veins (small arrows); c – Susceptibility Weighted Image showing very low signal within a thrombosed cortical vein in the right parietal area (arrow); d - MR venography without contrast injection showing a complete lack of signal in the thrombosed left transverse sinus (arrows).

subjects required 2, 3 or even 4 imaging examinations for the final diagnosis to be reached (Table 4).

The retrospective analysis of all neuroimaging examinations revealed CVT of the superficial venous system in all subjects with a coexisting thrombosis of the deep venous system in one case (Tables 1–3). In the superficial CVT, thrombosed vessels were found bilaterally in 3 patients, exclusively on the left side in 4 patients and on the right side in 4 patients. In the superficial CVT, the thrombosed vessels were superior sagittal sinus (6 patients), transverse sinus (9 subjects), sigmoid sinus (9 subjects) and internal jugular vein (8 subjects) (Fig. 2, 3). In 7 subjects, cortical veins were also thrombosed such as the vein of Labbé (3 patients), the vein of Trolard (2 patients), small convexity veins (3 patients) and small infratentorial cortical veins (1 patient) (Tables 1–3, Fig. 3 a–c). Parenchymal lesions due to superficial CVT were found in 9 patients, including hemorrhagic infarctions in 6 patients (Table 3) and non-hemorrhagic lesions in 3 subjects (Table 2, Fig. 1). One subject developed bilateral cerebral lesions. In 2 subjects, no cerebral lesions were found in the course of superficial CVT (Table 1). In the case of the deep CVT, deep veins and the straight sinus were thrombosed with the non-hemorrhagic lesions involving the left basal ganglia region, left thalamus and the periventricular white matter (Table 2, Fig. 1b).

Follow-up neuroimaging examinations showed no venous recanalization in 2 patients, partial recanalization in 3 subjects and total recanalization of the thrombosed vessels in 5 patients (Tables 1–3). In 1 subject, the status of the vessels was not possible to assess due to the lack of the dedicated vascular imaging. In all patients, brain lesions showed normal evolution in time.

Two patients with non-hemorrhagic lesions with full vessel recanalization showed complete or almost complete regression of the parenchymal changes. None of the non-hemorrhagic lesions underwent secondary hemorrhagic transformation. Despite the rate of recanalization, all hemorrhagic venous infarctions left post-malacic foci within brain parenchyma.

Correlation of the results of neurological and neuroimaging examinations

The mildest clinical symptoms (isolated headache) were observed in patients without any brain lesions and CVT of internal jugular veins and transverse and sigmoid sinuses. The worst clinical course and outcome were noted in 2 subjects with hemorrhagic infarctions and CVT of the superior sagittal sinuses. Seizures and hemiparesis

(with or without accompanying headache) were strongly associated with the existence of focal brain lesions. Patients with non-hemorrhagic lesions showed either seizures or hemiparesis, not both, while patients with hemorrhagic lesions usually showed both seizures and hemiparesis (4 out of 6 subjects), apart from 2 cases in which either seizures or hemiparesis were present. Seizures were noted in 1 out of 3 (33%) subjects with non-hemorrhagic lesions and in 5 out of 6 (83%) subjects with hemorrhagic lesions. In all subjects with seizures, brain lesions were located within the cortex of the frontal lobes. Hemiparesis was seen in 2 out of 3 (66.6%) subjects with non-hemorrhagic cortical lesions and in 5 out of 6 (83%) subjects with hemorrhagic cortical brain lesions (Tables 1–3).

Full recovery without any neurological symptoms was observed in all subjects without any brain lesions, in 1 out of 3 subjects (33%) with non-hemorrhagic brain lesions in the deep structures and in 1 out of 6 (16.6%) subjects with hemorrhagic lesions located in the right occipital lobe. Secondary epilepsy was observed in 1 patient with non-hemorrhagic cortical lesions, even though it disappeared completely in the follow-up examinations, and in 4 patients with hemorrhagic infarctions and post-malacic foci within the cortex. Persistent hemiparesis was observed in 1 subject with non-hemorrhagic infarction and in 3 subjects with hemorrhagic lesions and post-malacic foci within the cortex (Tables 1–3).

A coexistence of the superficial and deep CVT did not worsen the neurological state of the patients who fully recovered after the treatment.

There was no direct association between the rate of recanalization of the thrombosed vessels and the neurological recovery. Out of 5 patients with total vessel recanalization, 2 subjects showed full neurological recovery and the other 3 with non-hemorrhagic and hemorrhagic brain lesions developed symptomatic epilepsy. Out of 3 subjects with partial vessel recanalization, 2 fully recovered and 1 patient with hemorrhagic brain lesion developed symptomatic epilepsy and hemiparesis. Two subjects with no vessel recanalization and brain infarcts developed persistent hemiparesis and seizures (Tables 1–3).

There was no direct association between the delay in the onset of the thrombolytic treatment and neurological outcome. In patients who fully recovered, the onset of the treatment ranged between 1 and 13 days (mean 5.25 days), and in patients who developed persistent neurological symptoms it ranged between 1 and 21 days (mean 7.4 days).

Discussion

The clinical picture of CVT is usually non-specific and may mimic other neurological pathologies. In the presented group of patients, the most frequent symptoms were also non-specific and included headache, seizures and hemiparesis. Headache is reported to be the most frequent initial symptom of CVT, which was also noted in the group of our patients and can be either isolated or accompanied by other symptoms. Isolated headache is not so common (reported in 14% of cases) and was present in 1 of our patients.[12] It is a diagnostic challenge and it should always raise the suspicion of CVT, especially in patients with high risk factors. An analysis of risk factors may be helpful in emergency settings, especially history of estrogen treatment and peripartal period in young women. Neoplastic disease or head trauma, which were major risk factors also found in our patients, should raise the suspicion of CVT.

It has to be stressed that there are no laboratory findings or tests that would be easy to do in emergency settings and could confidently rule out CVT. The value of D-dimers was tested in several studies and was reported to be normal in 4–26% of CVT patients.[13,14] In our study, these numbers are even higher since the elevated D-dimer concentration was noted in only 5 out of 11 patients (45%), which makes this test unreliable for confirming or rejecting the diagnosis of CVT. After the diagnosis of CVT is established, it is important to perform testing for congenital thrombophilia. The presence of congenital thrombophilia potentiates the risk of CVT, which was also the case in our 3 female patients who developed CVT due to combined abnormalities of the thrombotic system and either oral contraception or cesarean section.[1] It is important to diagnose congenital thrombophilia to protect the patient from future potential CVT incidents and to look for this disorder in the patient's family members so that preventive measures can also be taken in this group.

Since the clinical picture including symptoms and laboratory findings is often very non-specific, the role of neuroimaging is crucial to raise the suspicion of CVT. Usually the first imaging method is a CT examination of the brain. It has to be stressed that, similarly to the non-specific neurological symptoms, CT images may also be difficult to interpret and pose a diagnostic problem which was also noted in the cases of our patients.

According to the literature, only in 25% of cases can CVT be visualized with an unenhanced CT as an increased density within a thrombosed vein.[8,15] A retrospective detailed evaluation of our cases showed these signs in 8 out of 11 emergency CT examinations but only in 2 cases it was correctly reported. It has to be stressed that these signs may be difficult to spot, require an experienced radiologist and may be easily overlooked especially in the cases of CVT within small cortical veins, or subjects with elevated hematocrit and in the coexistence of subarachnoid hemorrhage or subdural hematoma. Administration of iodinated contrast agents improves the detection of a thrombus in CT and it helped to diagnose CVT in 2 of our subjects. The clot within a vessel is visible as a filling defect, which in the case of a thrombus within a superior sagittal sinus is known as

an empty delta sign.[8,15] The best technique for a direct detection of thrombosis using the CT technique is CT venography. It was used to diagnose CVT in 1 of our subjects after inconclusive contrast-enhanced CT. However, this examination is associated with a high dose of radiation and requires an iodinated contrast agent, which may sometimes cause serious side effects.

Changes within brain parenchyma in the course of CVT can be varied, ranging from small areas of decreased density to large hemorrhagic lesions. However, it has to be stressed that even in the course of CVT involving large venous sinuses, sometimes no evident lesions within the brain can be found in CT imaging.[1,8,9] This was the case in 2 of our patients with CVT of transverse and sigmoid sinuses in whom no visible changes within brain parenchyma were detected either in emergency CT or any of the follow-up neuroimaging examinations. In another 2 subjects, emergency CT examinations were reported normal since no cerebral lesions were visible initially. They appeared a few days later in the follow-up examinations and the only pathological findings in the retrospective analysis of the emergency CT examinations were hyperdense deep veins in one case and a hyperdense single cortical vein in the other case, which were overlooked in the emergency settings.

Suspicion of CVT should always be raised by bilateral hypodense areas within deep structures such as thalamus and basal ganglia, which are typical of deep CVT.[8] Other cerebral lesions with high suspicion of CVT are hemorrhagic lesions in the brain, located peripherally within the cortex, which do not correspond to the territories supplied by arterial vasculature, and especially when they are bilateral.[8] On the other hand, such hemorrhagic lesions are often confused with hemorrhagic arterial infarcts, cerebral contusions and bleeding due to a tumor or vascular malformations, which was also the problem in the group of our patients. In our study, none of the hemorrhagic lesions were correctly reported as hemorrhagic infarctions due to CVT.

In the case of suspected CVT based on a standard CT scan, the next examinations which could confirm the diagnosis may be CT venography or MRI. In 3 of our patients, it was the contrast enhanced MRI which ultimately diagnosed CVT. Parenchymal changes are better visible in MRI than in CT.[9] In addition to better detection of brain lesions, MRI allows for an unambiguous differentiation of venous from arterial strokes, since the latter show diffusion restriction in DWI.[16] Another advantage of MRI is its capability to image thrombosed veins and venous sinuses, even in the examinations without contrast application, with greater sensitivity than unenhanced CT examinations, and in the case of thrombosis of cortical veins with greater sensitivity than CT venography.[8] In unenhanced MRI, the signal intensity of venous thrombi varies according to their age and is related to the paramagnetic effects of the products of hemoglobin breakdown. A venous thrombus in the acute stage (0–5 days) may be very poorly visible, while in the subacute stage its signal is predominantly hyperintense on all images (T1, T2, FLAIR, DWI), which is associated with the presence of methemoglobin.[1,8] Hyperintense veins are observed in over 50% of patients with CVT. MRI without contrast administration is a particularly useful tool for the detection of thrombosis of small cortical veins, especially with the use of T1-weighted, FLAIR, T2* gradient or susceptibility weighted images (SWI).[1,17] Administration of the contrast agent in MRI, similar to CT imaging, improves the detection of CVT. MR venography may be performed with or without administration of contrast medium. In MR venography without contrast administration, chronic, peripherally located thrombi or partially recanalized thrombi with preserved blood flow within the sinus will not be visible. In such cases, the best imaging technique will be MR venography with contrast injection or CT venography, which show a chronic thrombus as a filling defect within a contrasted vessel.[18,19]

At present, DSA, which is still regarded as the gold standard for diagnosing vascular pathologies, is only rarely performed in CVT because of its invasiveness.[9] It is only used in diagnostically difficult cases. In our study, DSA was performed in 2 cases. In 1 patient, DSA was performed to reach the final diagnosis of CVT. In this case, venous thrombosis included the anterior aspect of the superior sagittal sinus, which was not seen in the earlier performed examinations such as CT, MRI with contrast and CT venography, even in the retrospective analysis. It the other patient, DSA was performed after the false suspicion of the bleeding vascular malformation based on an emergency CT examination. It has to be stressed that initial radiological diagnosis has an impact on further diagnostic procedures and the selection of subsequent imaging examinations, as shown in Table 4. In our study, a radiological suspicion of a vascular malformation resulted in DSA, which is an invasive procedure and could have been abandoned in this case.

The other aim of our study, apart from the analysis of the clinical and radiological diagnostic challenges, was to analyze the associations between the clinical course and outcome of the patients and the location of the venous occlusion, type of parenchymal lesions and rate of vessel recanalization.

In our study, all patients developed thrombosis of the superficial venous system, while only 1 person (9%) also exhibited features of deep venous system thrombosis. This is consistent with the literature reports, in which the incidence of CVT of the superficial system, including the dural sinuses and cortical veins, is estimated to be approx. 67%, and in the case of the deep system, including internal cerebral veins, vein of Galen and the sinus rectus, at about 32%.[8] According to the literature, the most often involved sinus is the superior sagittal sinus (63%), followed by the transverse sinus (57%), then sigmoid and

rectus (each 15%).[8] In our material, CVT of transverse and sigmoid sinuses was the most frequent (80%), followed by superior sagittal sinus (40%) and rectus (20%). An interesting observation in our study is the high percentage (55.5%) of thrombosis of cortical veins. In the literature, thrombosis of cortical veins is estimated at around 6%, but in our opinion this low percentage is associated with an underestimation of this phenomenon due to difficulties with imaging thrombosis within these small vessels. In our study, we noticed that coexistence of the deep venous thrombosis did not worsen the clinical course of the patient, who presented with mild symptoms and in the end fully recovered without any persisting neurological symptoms. According to the literature, when the deep cerebral venous system is occluded, the clinical picture is usually more severe with coma and motor deficits, which are usually bilateral, though more limited thrombosis of the deep venous system without parenchymal lesions can cause relatively mild symptoms.[20] Our patient with deep CVT developed parenchymal lesions but they were not hemorrhagic and disappeared completely in the follow-up examinations.

In our study, the type of cerebral lesions had the strongest influence on the clinical outcome of the patients. In the course of CVT, brain hemorrhagic lesions occur more frequently than non-hemorrhagic.[8] They leave areas of malacia or cavities in the brain tissue, which have an impact on the occurrence of late clinical symptoms. In our study, 5 out of 6 subjects with hemorrhagic brain lesions developed either persistent secondary epilepsy, hemiparesis or both. The only person with hemorrhagic lesions who fully recovered was the male subject with parenchymal lesion in the right occipital lobe and the symptoms of transient visual phenomena and mild left-sided hemiparesis. It has to be stressed that non-hemorrhagic changes in the brain in the course of CVT may be either true irreversible necrotic lesions or areas of edema which may resolve completely.[8] In patients with real irreversible venous infarcts, neurological defects are reported to be more frequent.[3] In our study group, 2 patients showed edematous lesions which resolved completely in the follow-up examinations – in one case without leaving any persistent neurological symptoms (patient with deep CVT), while in the other case causing persistent secondary epilepsy, probably due to irreversible damage of the frontal cortex not visible in the follow-up imaging examinations.

In our study, we also looked at the correlation of the rate of vessel recanalization and the clinical outcome. Thrombus recanalization occurs at different rates in patients, which is reported to have an impact on clinical symptoms and outcome. Endogenous thrombolysis often coexists with the processes of thrombosis, which is reflected by the fluctuating course of clinical symptoms in CVT.[21] Complete recanalization occurred in 5 and partial recanalization in 3 patients. Total recanalization was not necessary for a very good final outcome. In our study, 2 out of 3 subjects with only partial recanalization showed full neurological recovery and 3 with total recanalization but also with parenchymal lesions were left with persistent neurological deficits. Thus, in our opinion, the factors which influence the clinical outcome the most are the presence and type of cerebral lesions.

Conclusions

Due to non-specific clinical symptoms and the results of laboratory tests as well as very diverse radiological appearance, the diagnosis of CVT is still a great challenge for neurologists and radiologists. Young age, female gender and risk factors such as oral contraceptives, cancer or cranio-cerebral trauma as well as non-specific symptoms such as headache or seizures should always arouse a clinical suspicion of CVT. Neuroimaging is of crucial importance for the final diagnosis and clinical outcome of the patient. The most difficult and challenging patients are those with discrete radiological symptoms, easy to overlook, or with brain lesions mimicking other entities, e.g., tumors, contusions, arterial ischemia or vascular malformations. Suspicion of CVT should be raised by hemorrhagic lesions, particularly located within the cortex or bilaterally within the deep structures. After emergency CT, MRI is the next examination recommended to confirm the diagnosis, especially in the case of young people or in the subacute phase of CVT. In the chronic phase, CT or MR venographies are the best methods to assess the patency of thrombosed vessels. The factor which influences the clinical course and outcome the most is the location and type of brain lesions, with hemorrhagic cortical infarctions bringing the worst prognosis and being associated with the highest rate of persistent neurological deficits. In our opinion, quick diagnosis before parenchymal hemorrhagic lesions are visible on CT is of crucial importance and requires a constant alertness and good cooperation of neurologists and radiologists, especially in emergency settings.

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Accumulation of mutations in reverse transcriptase of hepatitis B virus is associated with liver disease severity in treatment-naïve Chinese patients with chronic hepatitis B

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Abstract

Background. Mutations in reverse transcriptase (RT) of the hepatitis B virus (HBV) are demonstrated to be strongly associated with nucleos(t)ide analog resistance, which is supposed to be the biggest obstacle during the long-term anti-viral treatment of chronic hepatitis B. However, the presence of RT mutations in treatment-naïve chronic hepatitis B patients and its clinical significance are not well known.

Objectives. To investigate the significance of mutations in reverse transcriptase of the hepatitis B virus in treatment-naïve Chinese patients with chronic hepatitis B.

Material and methods. In this study, 288 treatment-naïve chronic HBV patients were recruited and the RT region was sequenced. The results showed that 71 patients (24.65%) were found with RT mutations, within which there were no well-defined primary nucleotide analog-resistant mutations.

Results. There were a total of 28 mutant sites, which formed 3 dominant mutant clusters: rt124-139, rt191-212 and rt225-229. Among these 71 patients, 63.38% (45/71) of patients had a single mutation while 19.72% (14/71), 12.68% (9/71) and 4.23% (3/71) of patients had 2, 3 or 4 mutations, respectively. Patients with RT mutations showed significantly decreased serum baseline HBV DNA loads ($p = 0.0363$) and blood platelet count ($p = 0.0181$) than patients without RT mutations. Patients with multiple mutant sites (≥ 2) had significantly decreased baseline HBV DNA loads ($p = 0.0004$) and blood platelet count ($p = 0.0011$) than patients with single mutant site. Moreover, the number of RT mutant sites is significantly associated with severity of liver fibrosis ($p = 0.0128$).

Conclusions. This study demonstrated that there was a prevalence of RT mutations in treatment-naïve chronic hepatitis B patients, which reflects a tougher liver environment for the virus and deeper liver injury for the host. Accumulation of RT mutations was associated with liver disease severity in treatment-naïve chronic hepatitis B patients.

Key words: hepatitis B virus, mutation, treatment-naïve, reverse transcriptase

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Hepatitis B virus (HBV) infection is the most common etiologic agent of acute and chronic liver disease in China.[1] Individuals infected with HBV in infancy or childhood often develop into chronic hepatitis, eventually progressing from liver fibrosis to cirrhosis or hepatocellular carcinoma.[2] HBV is an enveloped, partially double-stranded DNA virus containing a genome of 3200 nucleotides encoding four open reading frames (ORFs): pre-S/S, pre-C/C, HBX and polymerase. The polymerase gene includes four domains such as the terminal protein, spacer, ribonuclease H and reverse transcriptase (RT). The RT replicates the HBV genome through its DNA polymerase activity using RNA intermediates as a template.[3] Because the RT does not possess proof reading activity during viral replication, the error rate of HBV genome synthesis has been mounted as 10^{-7} per nucleotide, which is 10-fold higher than other DNA viruses.[4] The high rate of HBV mutation results in lots of genomic variants and survival of the fittest during the selection of liver environment, anti-viral drugs and host immune defense.

Mutations in the RT region were thoroughly reported in recent data for their nucleos(t)ide analog-resistant activity. For instance, rtM204I is a classical mutation reducing susceptibility to monotherapy by lamivudine (LMV) or telbivudine (LdT); the rtI169T mutation was reported as an entecavir (ETV)-resistant amino acid substitution and rtA181V showed its resistance to adefovir dipivoxil (ADV).[5–8] In addition, gene mutations A799G, A987G, and T1055A in the RT region have been reported consistently associated with hepatocellular carcinoma (HCC) and these mutations were always detectable 4–5 years prior to HCC diagnosis.[9] Moreover, since the envelope (S) gene is completely overlapped by the RT gene, mutations in the RT region may produce changes in its overlapping S gene, causing amino acid substitution or stop codon in the S protein. For instance, rtM204I and rtL180M/M204I produce I195M and W196S in the S protein, leading to an altered structural integrity of S protein and consequently reduced its binding affinity to anti-HBs antibody.[10]

Compared with single amino acid mutation, a combination of 2 or more mutations is complicated and always linked to the development of HCC. A combination of G1613A + C1653T or any pre-S mutation + C1653T + T1753V significantly associates with hepatocellular carcinoma in genotype C HBV infected patients.[11,12] Among the 8 key mutations comprising G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A and G1899A in the X/preC region, a combination of ≥6 mutations shows increased risk of HCC in genotype C2 HBV infected Korean patients.[13] However, the combination of mutations in the RT region and its clinical significance have seldom been discussed.

In this study, we sequenced the RT gene of HBV in 288 treatment-naïve chronic hepatitis B patients and 71 patients (24.65%) were found with RT mutations. The pattern of mutations and nucleotide analog-resistant mutations as well as the combination of these mutations were analyzed.

Material and methods

Patients and blood samples

A total of 288 treatment-naïve chronic HBV patients were enrolled at the First Affiliated Hospital of Xinxiang Medical University (Henan, China) from July 2009 to May 2014. All patients were diagnosed as chronic hepatitis B based on the criteria suggested by the Chinese Medical Association for Liver Diseases in 2005.[14] The average duration time of HBV infection since first diagnosed as serum HBsAg positive was 28.71 ±10.24 years. They were all confirmed LMV, ADV, ETV, LdT and interferon (IFN) treatment-naïve. Patients co-infected with the hepatitis A/C/D virus or human immunodeficiency virus, or other concomitant liver disease such as autoimmune liver disease, primary biliary cirrhosis, alcohol or drug abuse were excluded. All patients had written consent on entry into the trials and agreed to authorize the hospital to deal with their blood and tissue samples for research purposes. The study was approved by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University. Patients' sera were collected and stored at -80°C.

Diagnostic tests

Liver function tests and serum HBV markers were conventionally conducted in the clinical lab of the First Affiliated Hospital of Xinxiang Medical University. Serum hepatitis B s antigen (HBsAg), anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe and anti-HBc were determined on the wholly automatic immune fluorescence analyzer Abbott Type I2000 (Abbott Laboratories, USA) using the original, attached commercial kits. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) were assayed by the wholly automatic biochemical analyzer DXC800 (Beckman Coulter, USA). Blood platelet count was determined on the fully automatic hematology analyzer LH780 (Beckman Coulter, USA). Serum HBV DNA loads were quantified with fluorescence quantitative PCR assay (Da'An GENE, Guangzhou, China) performed on an ABI 7500 (Applied Biosystems, USA). The detection sensitivity was as low as 500 copies/mL.

Amplification and sequencing of HBV RT region

HBV DNA was extracted from 500 µL sera of the patients according to the protocol of the QIAamp DNA Blood Kit (Qiagen, Germany). The HBV RT gene was amplified by nested PCR as previously described.[15]

The primers used for the outer round PCR system were 5-AGTCAGGAAGACAGCCTACTCC-3 (nt 3146-3167) and 5-AGGTGAAGCGAAGTGCACAC-3 (nt 1577-1596); the primers used for the inner round PCR system were 5-TTCCTGCTGGTGGCTCCAGTTC-3 (nt 54-75) and 5-TTCCGCAGTATGGATCGGCAG-3 (nt 1258-1278). PCR conditions were 94°C for 3 min; 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min; then 72°C for 10 min. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Germany) and directly sequenced (Sangon Bioengineering, Shanghai, China). Nucleotide sequences were analyzed using DNAStar 5.0 and MEGA 4.0 software. Mutations of the HBV RT gene were determined by sequence alignment with the reference stains in GenBank.[16]

Fibrosis staging

Liver biopsies were obtained using a 17G core aspiration needle (Hepafix, Germany), with a biopsy length of 2~5 cm. The biopsy specimens were fixed, paraffin-embedded, cut into 3~5 μm thick sections, and stained with haematoxylin and eosin (H&E) and Masson's trichrome. The degree of liver fibrosis was evaluated by experienced hepato-pathologists, who were blinded to the clinical data of the patients. Staging of liver fibrosis was performed semi-quantitatively according to the published grading and staging system: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.[17]

Statistical analysis

Statistical analyses were performed using SPSS16.0 software (SPSS, Chicago, USA). The one-way ANOVA t-test or Pearson's χ^2 test were used appropriately for continuous variables and categorical variables. The serum level of HBV DNA loads were expressed on a logarithmic scale. A p-value of less than 0.05 was considered to be statistically significant.

Results

Patient characteristics

The HBV RT gene was sequenced in all 288 treatment-naïve chronic hepatitis B patients. The HBeAg positive rate was 71.18% (205/288), and all of them were genotype B or C with the HBV B/C ratio of 38.19%/61.81% (110/178). Multiple comparisons of the main characteristics according to serum HBeAg positivity are shown in Table 1. HBeAg-negative patients were significantly older than the HBeAg-positive group, while no significant differences in gender, genotype, ALT, AST and TBIL were found between the HBeAg-positive and HBeAg-negative group.

Table 1. Characteristics of 288 treatment-naïve chronic hepatitis B patients

Characteristics	HBeAg+ CHB (N = 205)	HBeAg– CHB (N = 83)	p-value
Age (years)	42.12 ±14.90	46.56 ±13.61	0.0169
Gender (male/female)	131/74	56/27	0.5655
Genotype (type B/type C)	79/126	31/52	0.8510
HBV DNA (log copies/mL)	6.67 ±1.42	5.49 ±1.33	<0.0001
ALT (U/L)	660.26 ±782.94	504.30 ±546.09	0.0984
AST (U/L)	353.41 ±376.74	281.61 ±261.42	0.1135
TBIL (μmol/L)	74.76 ±102.23	73.83 ±100.86	0.9441
Platelet ($\times 10^9$/L)	169.44 ±159.85	122.31 ±55.58	0.0093

HBeAg – hepatitis B e antigen; HBV – hepatitis B virus; ALT – alanine aminotransferase; TBIL – total bilirubin.

HBeAg-negative patients had much lower serum viral loads than HBeAg-positive patients (HBeAg$^+$ vs HBeAg: $10^{6.67}$ vs $10^{5.49}$, $p < 0.0001$), which is consistent with published data.[18] Interestingly, HBeAg-negative patients also showed a significantly lower level of blood platelet than HBeAg-positive patients ($p = 0.0093$).

Characterization of mutations in HBV RT gene from treatment-naïve CHB patients

Among 288 treatment-naïve chronic hepatitis B patients, HBV RT mutations were found in a total of 71 patients (24.65%). The distribution of amino acid mutant sites within the RT gene identified in this study is shown in Fig. 1. There was a total of 28 mutant sites, which formed 3 dominant mutant clusters: rt124-139, rt191-212 and rt225-229, indicating different susceptibility to mutations under a natural history of HBV replication. Although there were 3 patients that had mutations at the rt181 site (A181S), no well-defined primary nucleotide analog-resistant mutations (i.e. I169T, A181T/V, T184A/C/F/G/I/L/M/S, A194T,

Fig. 1. Amino acid substitutions at 28 positions of HBV reverse transcriptase analyzed in this study

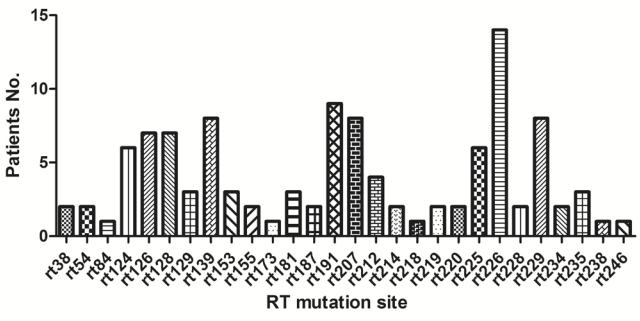
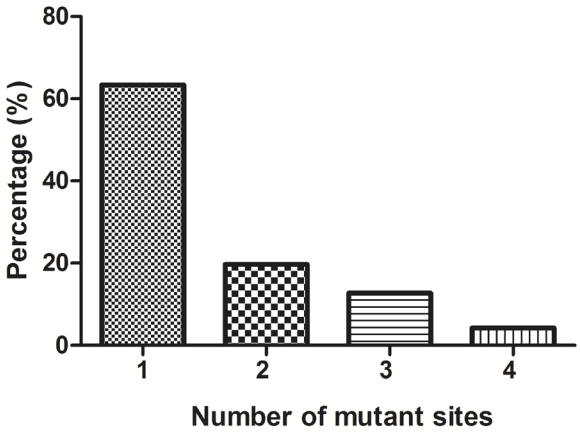


Fig. 2. Frequency of accumulation of amino acid substitutions in 71 patients with RT mutations



S202C/G/I, M204I/V/S, N236T, M250I/L/V) were found among these treatment-naïve patients.[19,20] When analyzing the number of mutations in these 71 patients with a mutant RT gene, we found that 63.38% (45/71) patients had a single mutation while 19.72% (14/71), 12.68% (9/71) and 4.23% (3/71) of patients had 2, 3 or 4 mutations, respectively (Fig. 2).

Correlation between HBV RT mutations and clinical features

To investigate the clinical significance of HBV RT mutations in treatment-naïve chronic hepatitis B patients, the clinical characteristics were compared between the 217 patients without RT mutations and the 71 patients with RT mutations. No significant differences were found in age, gender, HBV genotype, HBeAg status, ALT, AST and TBIL between patients with and without RT mutations (Table 2). However, patients with RT mutations showed significantly decreased serum baseline HBV DNA loads (p = 0.0363), indicating a much tougher environment for viral survival or replication. Furthermore, there was a much lower blood platelet count in patients with RT mutations (p = 0.0181), demonstrating that there may be much deeper liver injury in these patients.

Correlation between clinical features and number of RT mutations

Among these 71 patients with RT mutations, 45 patients (63.38%) had a single mutant site and 26 patients (36.62%) had 2 or more mutant sites. A comparison of clinical characteristics between these 2 groups is summarized in Table 3. Although there were no significant differences in age, gender, HBV genotype, HBeAg status, ALT, AST and TBIL, the patients with multiple mutant sites (≥2) had significantly decreased baseline HBV DNA loads (p = 0.0004) and lower blood platelet count (p = 0.0011), compared to patients with a single mutant site. It demonstrated that there may be much deeper injury in the liver of patients with multiple RT mutations and the liver environment of these patients was not good enough for viral survival or replication.

Table 2. Comparison of main characteristics of patients with and without HBV RT mutations

Characteristics	Group with mutations (n = 71)	Group without mutations (n = 217)	p-value
Age (years)	45.17 ±11.72	42.83 ±14.91	0.2289
Gender (male/female)	48/23	139/78	0.5863
Genotype (type B/ type C)	26/45	84/133	0.7531
HBeAg (positive/negative)	51/20	154/63	0.8891
HBV DNA (Log copies/mL)	6.02 ±1.28	6.43 ±1.47	0.0363
ALT (U/L)	561.36 ±613.59	632.97 ±794.18	0.4878
AST (U/L)	312.34 ±298.72	339.38 ±382.13	0.5868
TBIL (μmol/L)	69.61 ±98.86	76.09 ±104.07	0.6452
Platelet (109/L)	125.14 ±46.39	165.91 ±141.87	0.0181

HBeAg – hepatitis B e antigen; HBV – hepatitis B virus; ALT – alanine aminotransferase; TBIL – total bilirubin.

Multiple RT mutations are associated with more severe liver fibrosis

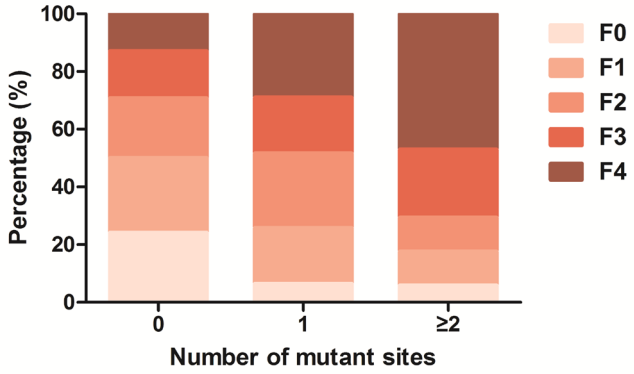
To investigate the relationship between RT mutations, especially multiple RT mutations, with liver disease se-

Table 3. Comparison of main characteristics of patients with different number of RT mutations

Characteristics	Single mutant site (n = 45)	Mutant sites (≥2) (n = 26)	p-value
Age (years)	45.17 ±11.72	42.83 ±14.91	0.2289
Gender (male/female)	48/23	139/78	0.5863
Genotype (type B/ type C)	26/45	84/133	0.7531
HBeAg (positive/negative)	51/20	154/63	0.8891
HBV DNA (Log copies/mL)	6.02 ±1.28	6.43 ±1.47	0.0363
ALT (U/L)	561.36 ±613.59	632.97 ±794.18	0.4878
AST (U/L)	312.34 ±298.72	339.38 ±382.13	0.5868
TBIL (μmol/L)	69.61 ±98.86	76.09 ±104.07	0.6452
Platelet (109/L)	125.14 ±46.39	165.91 ±141.87	0.0181

HBeAg – hepatitis B e antigen; HBV – hepatitis B virus; ALT – alanine aminotransferase; TBIL – total bilirubin.

Fig. 3. Distribution of liver fibrosis in 164 treatment-naïve chronic hepatitis B patients who had fibrosis staging performed based on the number of HBV RT mutations



F0 – no fibrosis; F1 – portal fibrosis without septa; F2 – portal fibrosis and few septa; F3 – numerous septa without cirrhosis; F4 – cirrhosis.

verity in treatment-naïve chronic hepatitis B patients, a total of 164 individuals had liver biopsies performed: 116 patients without RT mutations, 31 patients with a single RT mutation, and 17 patients with multiple RT mutations. Liver fibrosis was evaluated semi-quantitatively: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.[17] The distribution of liver fibrosis staging is showed in Fig. 3: the number of RT mutations is significantly associated with the severity of liver fibrosis ($p = 0.0128$). 24.14% (28/116) of patients without RT mutations, 6.45% (2/31) of patients with a single RT mutation, and 5.88% (1/17) of patients with multiple RT mutations were diagnosed as F0 fibrosis (no fibrosis). 12.93% (15/116) of patients without RT mutations, 29.03% (9/31) of patients with a single RT mutation, and 47.06% (8/17) of patients with multiple RT mutations had histological evidence of cirrhosis (F4 fibrosis).

Discussion

Reverse transcriptase (RT) preforms the major enzymatic activity for viral replication and the main target of anti-HBV drugs such as nucleos(t)ide analogs (NAs). NAs are reverse transcriptase inhibitors which mimic the natural nucleosides and incorporate into the DNA chain so as to inhibit viral replication.[21] Treatment of NAs is known as an effective way to restrain HBV replication and restore liver function. However, nucleoside analog-resistant mutations, which always occur at the RT region, are the biggest obstacle during NAs treatment. Moreover, nucleoside analog-resistant mutations were even found in treatment-naïve chronic hepatitis B patients, so it is suggested that patients should be examined for RT mutations before NAs treatment.[22] In this study, a total of 288 treatment-naïve chronic hepatitis patients were sequenced for the RT gene, and 71 patients (24.65%) were found with mutations. Among these mutations, no well-characterized primary or secondary/compensatory nucleoside analog-resistant mutations (i.e. I169T, A181T/V, T184A/C/F/G/I/L/M/S, A194T, S202C/G/I, M204I/V/S, N236T, M250I/L/V) were found according to the classification summarized by previous reports.[19,20] However, many mutations found in these treatment-naïve patients were putative nucleoside analog-resistant mutations, which may potentially associate with NAs resistance or compensatory replication capacity. For instance, rtV191I, rtV207I and rtL229V have been reported resistant to lamivudine, and rtA181S, rtV214A and rtE218D have been shown potentially related to adefovir resistance.[23–28] In any case, this data suggests that, rather than primary or secondary/compensatory nucleoside analog-resistant mutations, putative nucleoside analog-resistant mutations showed high prevalence in untreated Chinese chronic hepatitis B patients. The biological and clinical significance of these putative nucleoside analog-resistant mutations need to be further investigated.

The HBV RT gene consists of 6 functional domains (F, A, B, C, D and E) and 5 connecting interdomains (F–A, A–B, B–C, C–D and D–E).[29] In this study, we found 28 mutant sites in treatment-naïve patients, and these mutations didn't distribute evenly in the RT region. There were 3 dominant mutation clusters rt124-139, rt191-212 and rt225-229, located at the A-B interdomain, B-C interdomain/C domain and C-D interdomain, respectively. These mutation hotspots showed much a higher frequency of mutations than other sites, indicating that interdomains in the RT region are more susceptible to mutation under natural interaction between HBV replication and host immune defense.

As HBV infection usually starts in early childhood and lasts for a lifetime, the possibility of these patients getting HBV infection from their relatives who ever received formal antiviral treatment is very small, because during their childhood, LMV, ADV, ETV, LdT and IFN treatment were not popular in China. (The first anti-HBV drug LMV was approved by the FDA on 1998.) Since all the patients in this study were treatment-naïve and these RT mutations were selected under liver inflammation induced by the virus and host immune response, we compared the clinical data of patients with and without RT mutations and found that no significant differences were shown in age, gender, HBV genotype, HBeAg status, ALT, AST and TBIL. However, patients with RT mutations had significantly decreased serum baseline HBV DNA loads and much lower blood platelet. The decreased baseline HBV DNA loads reflected a tougher liver environment for viral survival and replication. Blood platelet count is an important index for liver fibrosis, and the much lower platelet count in patients with RT mutations indicated that there were fewer undamaged hepatocytes which can produce enough

thrombopoietin or there was more severe hypersplenism resulting in platelet activation and depletion.[30,31] In addition, decreased serum baseline HBV DNA loads and lower blood platelet were also found in patients with multiple mutant sites (≥2), compared to patients with a single mutant site. Therefore, the significance of RT mutations in treatment-naïve chronic hepatitis B patients is a reflection of a much tougher liver environment for both virus and host.

Compared to serum markers of liver function such as ALT, AST, TBIL and blood platelet count or other non-invasive measures such as aspartate aminotransferase-to-platelet ratio index (APRI), liver biopsy is considered to be the gold standard to evaluate the severity of liver fibrosis.[32,33] In this study, 164 chronic hepatitis B patients underwent liver biopsies before treatment. Liver fibrosis in patients with RT mutations was more severe than in patients without RT mutations, and liver fibrosis in patients with multiple mutant sites (≥2) was more severe than in patients with a single mutant site. This is consistent with the result of a decreased blood platelet count in patients with RT mutations, because patients with liver fibrosis are always associated with dramatically decreased blood platelet count.[34] The mechanistic explanation for the relationship between RT mutations and liver fibrosis is presently unclear. It could be speculated that the appearance of RT mutations was a sign of the interaction history between the HBV and host immune response in the liver environment. The appearance and accumulation of RT mutations reflected much longer or more severe liver inflammation, which plays a central role in the liver fibrosis of chronic hepatitis B patients.[35] This was the overall effect of natural occurring RT mutations on untreated patients, and the biological or clinical significance of individual mutations (especially those dominant mutations) needs to be further explored.

Based on this data, we demonstrated that the appearance and accumulation of RT mutations in treatment-naïve chronic hepatitis B patients was associated with decreased baseline HBV DNA loads and blood platelet count as well as more severe liver fibrosis. These results reinforce the linkage between the viral mutation and clinical progression of chronic hepatitis, and emphasize that the natural accumulation of RT mutations is a process of viral survival and chronic liver fibrosis.

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Clinical differences of *Helicobacter pylori* infection in children

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Abstract

Helicobacter pylori infection is widely spread all over the world. The prevalence of *H. pylori* infection in the world varies and depends on numerous factors such as age, ethnicity, geographical and socioeconomic status. Humans have been in a symbiotic relationship with this bacterium for thousands of years. However 10–20% of people infected with *H. pylori* are likely to develop gastroduodenal diseases such as peptic ulcer disease, iron deficiency anemia, gastric mucosal atrophy, metaplasia, dysplasia, MALT lymphoma, or gastric adenocarcinoma. Most of these diseases develop as the infection progresses and they are likely to occur later in life among the elderly. In the following years, the use of modern molecular techniques has led to the discovery of new *Helicobacter* strains and their genotypic differentiation. Newly discovered *Helicobacter* microorganisms can colonize human gastrointestinal tract and bile ducts. This article summarizes the distinct features of *H. pylori* infection in children including its prevalence, clinical manifestation, indications for treatment and recommended schemes of eradication.

Key words: children, treatment, epidemiology, *Helicobacter pylori*, clinical presentation

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H. pylori infection is the most common bacterial infection in the world and has a long history of maintaining a symbiotic relationship with people. Genetic studies suggest that *H. pylori* colonization in humans has occurred already 58,000 years ago and was initiated in east Africa resulting in its subsequent spread.[1] The discovery of *H. pylori* by Warren and Marshall in 1982 in patients with chronic gastritis and peptic ulcer disease has contributed to further major advances in *H. pylori* research.[2–6] In 1994, the World Health Organization classified *H. pylori* as a group 1 carcinogen for gastric adenocarcinoma.[6] In the following years, the use of modern molecular techniques has led to the discovery of new *Helicobacter* strains and their genotypic differentiation. Newly discovered *Helicobacter* microorganisms, such as *H. heilmannii, H. mustellae, H. felis, H. hepaticus, H. bilis*, and *H. pullorum* can colonize human gastrointestinal tract and bile ducts.[7–11]

Epidemiology of *H. pylori* infection

The prevalence of *H. pylori* infection in the world varies and depends on numerous factors such as age, ethnicity, geographical and socioeconomic status.[12–18] *H. pylori* infection is the most prevalent in developing countries of Africa, South America and Asia; whereas highly developed countries are the least infected. Goh et al. assessed the prevalence of *H. pylori* infection based on 31 publications from the Asia-Pacific region, Africa, Europe, and North and South America.[15] *H. pylori* serological tests were the most commonly used methods in determining the infection. The authors reported the high infection rates in the healthy population of South Korea (50.8%), Shanghai (71.7%), South Africa (66.1%), and the lowest rates in the the USA (7.5%) and Australia (15.5%), respectively. In developing countries, *H. pylori* infection is markedly more prevalent at a younger age than it is in developed countries.[13] According to Hunt et al., the prevalence of *H. pylori* infection in Ethiopian children, aged 2–4 years, was 48%, while in adults was over 95%; in Nigeria the prevalence of infection in children aged 5–9 years was 82%, while in adults ranged between 70–90%; in Mexico in children aged 5–9 years was 43%, and in adults ranged from 70 to 90%.[13] On the other hand, the *H. pylori* infection in children aged 5–18 years in Canada was reported to be 7.1%, whereas among adults aged 50–80 years to 23.1% respectively. In Europe, the most infected children were 1–17 years old and came from Bulgaria (61.7%), whereas the least infected children were from the Netherlands (1.2%). The epidemiological studies on *H. pylori* infection in Poland examined *H. pylori* seroprevalence and reported that 32.0% of children between 6 months and 18 years of age were infected, as compared to 84.19% of adults aged 19–89 years ($p < 0.0001$).[14] In addition, it should be noted that the prevalence of infection in children aged 2–3 years was as high as 26.4%.[14] Rowland et al. studied the prevalence of *H. pylori* infection in children in terms of age and infection risk factors.[17] The study included 327 children between the age of 24 to 48 months. *H. pylori* infection in children, parents and siblings was diagnosed using the carbon 13-urea breath test. Children without *H. pylori* infection were examined each year for 4 subsequent years with the urea breath test. Out of 327 children 28 were infected with *H. pylori;* and the average age of infected patients was 32.78 months. The rate of new infections per 100 persons a year in children aged 3–4 years were 4.20, whereas in children aged 4–5 years 2.07. Only 1 child aged 5–8 years was infected. This child's mother and older siblings were infected with *H. pylori*, and the child itself had been bottle-fed after 24 months of age, which was also considered a risk factor for infection. The results of these studies confirm that *H. pylori* infection occurs mostly in the first years of children's life and the risk of infection decreases when the children are over 5 years of age.

The most common risk factors of *H. pylori* infection in children were poor socio-economic and hygienic conditions and high population density.[14–16,19] According to Porras et al., the presence of 3 or more children in the family in addition to the lack of running water and sanitation, increases the risk of infection.[19] Moreover, Bostos et al., in their meta-analysis, demonstrated that children attending childcare facilities are more prone to frequent infections with *H. pylori*.[20] On the other hand, the main reasons for the presence of *H. pylori* infection in adults are socio-economic conditions, the level of education, smoking, alcohol drinking, and the lack of proper hygiene habits.[14] In the recent decades, the study of *H. pylori* infection rate has shown a decrease in the number of people infected. This is related to the improvement of socio-economic conditions, and frequent antibiotic treatment administered to cure other diseases.[21–24]

Kosunen et al., in a Finnish study, showed a fall in the seroprevalence rate from 56% to 31% between 1973 and 1994.[22] In addition, the prevalence of the pathogenic cagA(+) strains significantly decreased (34% to 8%, $p < 0.001$) more than in the case of cagA(–) strains (from 12% to 6%). The estimated seroconversion rate amounted to 0.4%, and the reversion rate to 0.13% per year.[23] Andreasson et al., in the prospective study carried out in Sweden, proved that for 23 years (1989–2012) the seroprevalence significantly fell from 38% to 16% (OR: 0.25; 95% CI: 0.11–0.59, $p = 0.001$, per decade).[24] The results from these studies confirm the cohort effect in developed countries. However, Rosenstock et al., in a Danish study, did not report a fall in the seroprevalence of *H. pylori* infection in 1983–1994.[25] In this study, the number of people infected with *H. pylori* was stable and ranged from 24.7% to 24.5%. It should be, however, noted that the reported *H. pylori* infection rate also depends on the detection methods. Serological methods give falsely higher

rates compared to the urea breath test or to the culture of *H. pylori* from stool samples as the methods can be related to *H. pylori* antibodies, presence for several months after eradication.

The incidence of pathogenic *H. pylori* strains cagA(+) in children has been also analyzed in various studies.[5,9,26,27] The occurrence of pathogenic *H. pylori* strains cagA(+) was more frequently reported in children than in adults (77.0% vs 63.8%, $p < 0.001$).[27] Significant differences in the percentage of cagA(+) strains in particular studies regions of Poland have been also confirmed ranging from 40.6% to 91.6%.[27] The most frequently observed genotypes in children were cagA(+) s1m1 (34.0%), and cagA(+) s1m2 (31.9%), whereas in adults cagA(+) s1m2 (31.0%) and cagA(+) s1m1 (23.1%). Statistically significant difference was reported between the prevalence of cagA(+) s1m1 in children and in adults (34.0% vs 23.1%, $p < 0.0279$).[27] However, in children *H. pylori* cagA(–) genotype was 23.0%, and in adults 36.2% ($p < 0.001$).

Infection transmission and reinfection

Urita et al. evaluated the potential relationship between *H. pylori* prevalence among children and grandparents in three-generation households in a Japanese rural town.[28] Based on this study, *H. pylori* spread in a 3-generation households occur not only through mother to child transmission but also through the grandmother to child route. Interestingly, having an infected father or grandfather was not a significant predictor for the infection being present in children.

The spontaneous resolution of infection or the infection with new strains primarily after eradication have been reported in people infected with *H. pylori.*[29,30] Vanderpas et al. reported that the reinfection after eradication for the period of 5 years amounted to 48.6%, and new infections that occurred in the period of 10 years in previously negative people amounted to 38.7%.[30] The risk of acquiring the infection was fourfold higher in non-European than in European children ($p < 0.001$). Other authors have also reported *H. pylori* seroconversion. Kumagai et al., in longitudinal cohort studies in children and adults between 1986 and 1997, reported *H. pylori* seroconversion rates were 1.1% and 1.0% per year for children and adults, respectively.[31]

Clinical presentation

H. pylori colonizes the gastric mucosa, leading to chronic inflammation in all infected humans, including children and adolescents. However, the majority of infected people have no statistically significant symptoms and remain free of symptoms throughout their lives.[14] Approximately 10–20% of people with *H. pylori* infection may develop stomach ulcers and/or duodenal ulcers, gastric mucosal atrophy, metaplasia, dysplasia, lymphoma, or gastric adenocarcinoma.[5,32–38] The specific presentation depends on the virulence features of the bacteria, host characteristics and environmental factors. Many studies have shown that the most virulent genotypes of *H. pylori* are cagA(+), vacA (+). The recently published large multicenter study analyzing genotypic and clinical differences of *H.pylori* infection in the Polish population demonstrated that the presence of cagA(+) s1m2 and cagA(+) s1m1 strains is found more frequently in children than in adults.[27] Genotype cagA(+) s1m1 has been detected statistically more often in children than in adults (34.0% vs 23.1%, $p = 0.02$). However, *H. pylori* cagA(–) s2m2 strain occurred more frequently in adults than in children (27.1 % vs 14%, $p = 0.003$). There was no effect of *H. pylori* infection on clinical symptoms such as nausea, regurgitation, vomiting, heartburn and abdominal pain in pediatric population.[27] In contrast, adults infected with *H. pylori* had more frequent episodes of heartburn and abdominal pain.[27]

Other studies demonstrated that in both children and adults, there is a statistically significant association between the seroprevalence of *H. pylori* and duodenal ulcer disease.[14,26] Biernat et al. analyzed the relationship between clinical outcomes of infection and the prevalence of *H. pylori* cagA, vacA, ICEA and babA2 genotypes in 130 children and adolescents aged 4–18 years.[26] The authors demonstrated a statistically significant association between the presence of cagA gene and duodenal ulcer ($p < 0.05$) whereas vacAs1/m1 genotype of *H. pylori* was frequent in children with gastritis and gastroesophageal reflux disease (GERD). In children with dyspepsia and GERD, vacAs2/cagA(–)/iceA1(+)/babA2(–) were frequent. Bontems et al. in a multicenter, prospective study enrolling 124 children from 11 European centers evaluated the risk factors for peptic ulcer and/or erosion of the stomach or duodenum.[39] The investigators demonstrated that *H. pylori* infection is the risk factor only for the occurrence of erosions and duodenal ulcers. In other studies the seroprevalence of *H. pylori* was more frequently observed in children than in adults.[27,40] The granulation of gastric mucosa associated with the lymphoid follicles hypertrophy and the thickening of the gastric mucosa folds were also seen. However, gastric mucosal atrophy, intestinal metaplasia and dysplasia occurred less frequently in children than in adults.[27,40]

De Sablet et al. have reported that *H. pylori* strain of European origin as compared to African origin was strongly predictive of the increased risk of precancerous gastric lesions, even in the low risk region.[37] Gonzalez et al. analyzed the gastric precancerous lesions in 312 patients.[38] The median follow-up was 12.8 years. The authors demonstrated advanced premalignant lesions in patients infected with *H. pylori* cagA(+) vacAs1/m1 strains

in comparison to patients infected with cagA(–) vacAs2m2 strains. This led to the conclusion that genotyping *H. pylori* strains may be useful in identifying higher risk patients infected with virulent strains of *H. pylori* that require more intensive surveillance.

Extra-intestinal manifestations

Many studies investigated the relationship between infection with *H. pylori* and iron-deficiency anemia, B12 deficiency, acute idiopathic thrombocytopenic purpura (ITP), and allergic diseases.[41–46] Infected children are less susceptible to wheezing, allergic rhinitis and skin allergies.[41] Several meta-analyses have confirmed the relationship between unexplained iron deficiency anemia and infection with *H. pylori,* both in children and adults.[42–44] Harris et al., in a prospective study, have demonstrated the correlation between *H. pylori* infection associated hypochlorhydia and the development of iron deficiency in 123 children.[45] Lu et al., evaluated the role of *H. pylori* in the pathogenesis of acute idiopathic thrombocytopenic purpura (ITP) and have shown that the prevalence of pediatric infections with *H. pylori* and controls ITP group were similar and amounted to 41.30% and 35.71%, respectively.[47] No difference was found between the initial platelet counts and megakaryocytes. Xiong et al., in a meta-analysis study, have demonstrated a possible link between *H. pylori* infection in Henoch-Schonlein purpura (HSP) as compared to the control group (49.27% vs 23.39%) in Chinese children.[48] Eradication of *H. pylori* infection reduced the recurrence of HSP. Wang et al., in a meta-analysis, have evaluated the association between asthma and *H. pylori* infection.[49] This analysis has shown a weak inverse association between asthma and *H. pylori* infection in children and adults. In contrast, Karimi et al. have not confirmed the relationship between *H. pylori* infection and asthma in children.[50]

Diagnosis of *H. pylori* infection in children

According to the guidelines from the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) diagnostic testing for *H. pylori* infection in children should be performed only when symptoms such as vomiting, persistent abdominal pain, and gastrointestinal bleeding can justify the gastroduodenoscopy with biopsy samples for examination.[51] In such cases it is important to determine the underlying cause of the symptoms and not solely focus on the presence of *H. pylori* infection. If there are no symptoms suggestive of organic disease, a decision whether to conduct diagnostic testing for *H. pylori* infection should be taken individually. Diagnostic testing for *H. pylori* infection may be considered in children with refractory iron-deficiency anemia, and idiopathic thrombocytopenic purpura.[51] Diagnostic testing for *H. pylori* infection is not recommended in children with functional abdominal pain.

A diagnosis of the infection should be based on invasive tests, i.e., culture, rapid urease test, polymerase chain reaction (PCR), or non-invasive tests, which include the urea breath test (UBT), detection of *H. pylori* antigens in stool or detection of antibodies to *H. pylori* in serum.[13,51–54] Diagnostic testing should be carried out at least 2 weeks after the completion of proton pump inhibitors (PPI) therapy, and 4 weeks after antibiotic treatment. The effectiveness of *H. pylori* eradication treatment is most commonly assessed with a non-invasive test, i.e., the urea breath test, or with *H. pylori* antigen stool test 4–8 weeks after eradication therapy. Serological tests measuring antibodies in serum or urine are not recommended for the evaluation of the treatment effectiveness, since antibodies can be present after *H. pylori* eradication for several months.[51]

Recommendations for *H. pylori* treatment in children

The main indication for treating *H. pylori* infection in children is peptic ulcer disease. *H. pylori* eradication should be considered in the case of its prevalence in the esophageal mucosa without peptic ulcer disease. First-degree relatives of patients with gastric cancer may be offered the eradication of *H. pylori*. A "test and treat" strategy is not recommended in case of children.[51]

Treatment of *H. pylori* infection in children

Studies on the effectiveness of *H. pylori* eradication are focused on selecting regimens which aim to achieve the highest eradication rates with the lowest incidence of side effects. The effectiveness of treatment regimens depends on the sensitivity of *H. pylori* strains to drugs used, the duration of the treatment and patients' compliance in taking medication.[51,53,54] It is recommended that drugs are tailored to the antibiotic susceptibility of *H. pylori* strains in a given region.[51,55–57] The best results of *H. pylori* eradication are achieved after the first treatment; subsequent treatments with the same antibiotic may be less effective due to the increasing secondary antibiotic-resistance of *H. pylori* strains. Clarithromycin has the most significant impact on the effectiveness of *H. pylori* eradication, followed by metronidazole. Low-level amoxicillin resistance (4.9–10.8%) has been reported in China,

Thailand and Korea; whereas high-level resistance to clarithromycin has been reported in European countries (15–24%) and in China (37.8%).[51,56–59] The prevalence of metronidazole resistance has been even higher.[21,51,57–62] In Poland, the resistance to clarithromycin depends on the region; in children it was between 9% and 26%, in adults from 3% to 27%; the resistance to metronidazole in children was from 16% to 43%, in adults from 27% to 52%.[27] However, it should be noted that in different countries *H. pylori* antibiotic resistance varies, and depends mainly on the frequency of antibiotics used for treatment of other infections in a given region.[21]

ESPGHAN and NASPGHAN recommend the following regimens in the first-line therapy:[51]

1. PPI (proton pump inhibitor) 1–2 mg/kg/day + amoxicillin (50 mg/kg/day) + metronidazole (20 mg/kg/day) or,
2. PPI + amoxicillin + clarithromycin (20 mg/kg/day) if the resistance to *H. pylori* does not exceed 15% in the region or,
3. Bismuth salts (bismuth subcitrate or subsalicytate) 8 mg/kg/day + amoxicillin + metronidazole.

The aforementioned medications may be administered in divided doses 2 times a day (with the maximum daily dose for amoxicillin amounting to 2000 mg, 1000 mg for metronidazole, 1000 mg/day for clarithromycin) for 10–14 days.[51] Sequential therapy, which includes dual therapy with PPI and amoxicillin for 5 days, followed by 5 days of triple therapy: PPI with clarithromycin and metronidazole/tinidazole has also been used in the last decade.[63–67] If *H .pylori* resistance to clarithromycin is high in the given region, PPI therapy in combination with bismuth and two antibiotics is recommended.

In the second-line therapy, if *H. pylori* are not eradicated with clarithromycin, a four-drug regimen with a new antibiotic is recommended. Moreover, determining the clarithromycin-susceptible *H. pylori* isolates is advocated.[51]

Conclusions

For thousands of years people have lived in symbiosis with *H. pylori*. Only some patients with *H. pylori* infection may develop health issues and life threatening diseases. This depends on the bacterial virulence, host characteristics, and environmental factors. Chronic infections with *H. pylori* can lead to peptic ulcer disease, iron deficiency anemia, MALT lymphoma, or gastric cancer. Children infected with *H. pylori* are less susceptible to wheezing, allergic rhinitis, and other allergic diseases. Indications for eradicating *H. pylori* infection in children are limited to peptic ulcer disease. A "test and treat" strategy does not apply in pediatric patients. Due to the increased *H. pylori* resistance to clarithromycin, its efficacy in *H. pylori* eradication is limited, and antimicrobial sensitivity testing is advocated.

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Hierarchical potential differentiation of liver cancer stem cells

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Abstract

Hepatocellular carcinoma (HCC) is one of the most malignant tumors in Chinese people and offers poor prognosis. Tumor tissue, like normal tissue, is hierarchically differentiated. Thus, minor tumor cell populations able to differentiate, such as stem cells, sustain tumor self-renewal and proliferation. The fact that liver cancer stem cells (CSCs) with different surface markers appear heterogeneous with respect to oncogenesis and drug resistance indicates that subpopulations of surface markers preserve the hierarchical potential of differentiation during proliferation, deterioration and relapse. The epithelial to mesenchymal transition (EMT) is correlated to tumor malignancy and aggression, and hepatocytes bearing EMT have obvious hierarchical differentiation potential with respect to signaling pathways such as transforming growth factor β, Wnt/β-catenin and microRNA. Therefore, it may be more effective for early diagnosis to monitor HCC recurrence using peripherally circulating CSCs, and these may also offer potential for HCC immunotherapy or for targeting HCC treatment using these markers. Thus, we reviewed the generation, hierarchical differentiation and clinical application of hepatic CSCs.

Key words: tumor heterogeneity, tumorigenesis, liver cancer stem cells, surface molecular markers

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Introduction

A high-grade malignant solid tumor with dismal prognoses, hepatocellular carcinoma (HCC) represents the 4th most common malignant neoplasm in Chinese people.[1] Although studies of HCC are advancing, methods for early and effective diagnosis of HCC require improvement.[2–3] Once HCC symptoms appear, the cancer is often very advanced and includes intrahepatic or systemic metastasis.[4] Poorly timed identification, late treatment and postoperative relapse contribute to high HCC mortality.[5] Although much effort to understand HCC recurrence has been put forth, the causes of therapeutic resistance are unclear. Cancer stem cells (CSCs), which may also be considered tumor initiating cells (TICs), may be the source from which HCC arises. In addition, CSCs may be the chief cause of tumor heterogeneity, which may contribute to drug resistance.[6–9] The hierarchical differentiation of hepatic CSCs and immunologic escape of self-monitoring are connected to tumorigenic stage.[6] Meanwhile, signaling pathways and genes that regulate stem cell differentiation may contribute to the control and maintenance of CSC differentiation, such as transforming growth factor β (TGF-β), Wnt/β-catenin and microRNA.[8–9] Thus, to better understand the etiology of HCC and address drug resistance, we must understand liver CSCs.

Research of the hierarchical differentiation potential of CSCs

Neoplasms are thought to represent the clonal proliferation of malignant tissue driven by genomic and genetic instabilities that are unrelated to mutation, selection or adaptation.[6] Recent data points to the importance of identification of genotypes among tumor patients because interpatient heterogeneity may drive treatment advances in oncology and may guide individualized therapies.[7] However, different genetic subclones have been identified in the same tumor and within the same pathological tumor classification with different genetic phenotypes. Then, over time, these subgroup clones behave differently, or cause tumor or intratumor heterogeneity, which are thought to be critical for tumorigenesis and metastasis.[8,9]

The generation of heterogeneity is related to the differentiation potential of embryonic stem cells that develop into various functional organs.[9] Thus, HCC is a stem cell abnormality and their ability to self-renew and differentiate is one of their most critical features, hence their reclassification as CSCs or TICs. These cells can initiate tumor formation after administration into nude mice, and they subsequently differentiate to different functional cancer cells in the lesions from which they are derived.

CSCs have 2 hypothetical contributions to oncogenesis and tumor heterogeneity. First, every tumor cell in the same tumor lesion is created with a potential for differentiation and a specific tumor cell population will proliferate and differentiate to stimulate tumor growth.[7] Next, the tumor lesion is hierarchically organized as is normal tissue differentiation from embryonic stem cells. Few CSCs can proliferate and sustain long-term tumor growth and the remaining tumor cells are silent due to a silencing of differentiation potential by the hierarchical organization.[7] Strictly speaking, CSCs have the same genotype as other tumor cells in the same tumor lesion, and are only unique after post-translational modifications and epigenetic events. Investigators have reported that this may be due to associations with the mechanism of normal tissue differentiation from embryonic stem cells.[10] All cancer cells originate from one precursor (the CSC) and in a tumor microenvironment, HCC progenitor cells acquire autocrine IL-6 signaling to stimulate growth and malignant progression for CSCs via upregulation of LIN28 expression.[11] Although all types of hepatic lineages can acquire oncogenic reprogramming to CSCs, how this occurs and why requires more study.[12–13]

Obtaining hepatic CSCs

The hepatic CSC theory has been promoted since 1989, and it suggests that a few specific hepatic cancer cell lesions obtain differentiation potential and cause malignant differentiation of HCCs by altering enzyme structures.[10] However, limited technologies at that time prevented direct data to support the existence of liver CSCs. Now, with immunofluorescence, immunomagnetic beads and flow cytometry, we can procure liver CSCs using liver cancer cell markers.[14] These methods also support research into mechanism of HCC heterogeneity and various hepatic CSC surface markers have been confirmed. At present, 10 hepatic CSC markers such as CD133, CD90, and CD44 have been reported.[15]

CD133, or Prominin-1, is highly expressed in fetal liver progenitor cells and is a member of the hematopoietic stem/progenitor cell transmembrane glycoproteins. CD133 is the earliest and most widely used protein for the separation of liver CSC subpopulations.[16–18] Stephanie reported that CD133-positive liver cancer cells were more tumorigenic than CD133-negative liver cancer cells in vivo and in vitro.[19] Also, stem cell-related genes such as Notch, β-catenin and Oct3/4 are highly expressed in the CD133-positive subpopulation.[20]

CD90, or Thy-1, is a surface marker of hepatic oval cells and CD45-negative CD90-positive subpopulations exists in hepatic tumor lesions and in the peripheral circulation.[20] Yang's group identified CD90-positive CD44-positive and CD45-negative markers for CSCs from liver lesions and the peripheral circulation, but they report that these stem cells have increased tumorigenicity and invasiveness compared to CD133-positive subpopulations.[21–23]

CD44 is a cell surface adhesion molecule that binds to extracellular ligand to regulate hepatic cancer proliferation, differentiation, metastasis and survival.[16,24–25] Moreover, CD45-positive subpopulations often express other stem cell markers such as CD133 and CD90.[21,26] Yang and co-workers suggest that CD44-positive liver CSC subpopulations are more aggressive than CD44-negative subpopulations.[21] Furthermore, Zhu's group reported that CD44-positive CD133-positive liver CSCs not only excessively express stem cell-related genes, but are more resistant to chemotherapy than CD44-negative CD133-positive cells.[26]

High expression of CD13, which is regarded to be a marker for liver cancer that is dormant or half dormant, occurs during the G1/G0 phase.[27–29] Haraguchi's group reported that CD13-positive hepatic cancer cells are more resistant to 5-FU and Adriamycin and have greater oncogenicity.[29] Also, CD13 expression was excessively induced by 5-FU and Adriamycin in liver cancer cells.[29]

Epithelial cell adhesion molecule (EpCAM), or CD326, is highly expressed in embryonic liver cells, bile duct epithelial cells, and hyperplastic bile duct epithelial cells in liver cirrhosis.[30] Yamashita et. al. reported that EpCAM is expressed more in liver cancer and the molecular signaling pathways of EpCAM-positive liver cancer cells are closely related to liver progenitor cells.[31–33] Simultaneously, Terris' group reported that EpCAM-positive AFP-positive liver cancer cells are more tumorigenic, mobile and invasive to the portal vein.[31] Therefore, EpCAM is thought to be a marker of liver CSCs.

OV6 is a marker of hepatic oval cells and is closely related to hepatic disease.[34,35] OV6-positive liver cancer cells are said to have more resistance to chemotherapy in vitro[36] and they are more aggressive in patients as well as are closely related to the clinical pathological characteristics of liver cancer.[37] However, OV6-positive patients with liver cancer are not unusually different with respect to disease-free and overall survival compared to OV6-negative patients with liver cancer.[37]

SALL4, a member of the zinc finger transcription factor family, is a stem cell marker.[38,39] Oikawa's group suggested that sal-like protein (SALL4) provokes the proliferation of liver cancer cells in vitro and induces overexpression of various liver CSC surface markers such as EpCAM, ATP-binding cassette-G2 (ABCG2) and CK19.[39] Others regard SALL4 to be a marker of liver CSCs, which can be used to predict HCC treatment outcomes.[40–42] Furthermore, expression of phosphatase and tensin homologue protein was suppressed and the formation of tumors in xenograft models was inhibited by overexpression of SALL4.[43] Thus, inhibiting expression of SALL4 can inhibit proliferation and differentiation of liver cancer cells.[44–48]

Many laboratories have reported that different liver cancer subpopulations sorted by surface markers have diverse features. For example, Zen and colleagues reported that liver cancer subpopulations sorted by ABCG2 have drug tolerance.[49] More interestingly, aldehyde dehydrogenase (ALDH) is not a stem cell surface marker but ALDH-positive CD133-positive cells have greater tumorigenicity and invasiveness.[50] Recently, toll-like receptors 4 and interleukin-6 were referred to as liver CSC markers due to their relationship with inflammation and tumorigenesis.[11,51] However, these subpopulations marked by different surface proteins maintain orderly differentiation potential during proliferation, deterioration and relapse in liver cancer and these may be the source of cancer heterogeneity. Thus, investigations of the mechanisms of liver CSC surface markers during cancer proliferation, metastasis and relapse may allow the development of agents that can inhibit tumor growth by targeting subpopulations of CSCs with unique differentiation potentials.

Mechanism of tumor heterogeneity produced by liver CSCs

Epithelial-to-mesenchymal transition (EMT) is closely related with the degree of liver cancer malignance and aggression, and liver cancer cells bearing EMT have obvious hierarchical differentiation potential closely associated with signaling pathways such as transforming growth factor β (TGF-β), Wnt/β-catenin and microRNA.[37,52,53]

Knock-out of TGF-β inhibits the incidence of liver cancer in nude mice and overexpression of TGF-β not only increases the oncogenicity of liver cancer via highly expressed transcription factors related to EMT in different tumor cells such as TWIST1, TWIST2, SNAI1, SNAI2, ZEB1 and ZEB2, but also makes the cells more likely to metastasize.[54] Also, E-cadherin is inhibited by overexpression of TGF-β and high expression of TWIST1 has been discovered in CD44-positive CD24-negative breast CSCs with more obvious potential for differentiation and more aggressive behavior.[55,56] In addition, TGF-β signaling alters the patterns of liver tumorigenesis induced by PTEN inactivation in liver CD133-positive CSCs but how tumor heterogeneity is regulated by TWIST1 is unclear.

Leal and colleagues reported that microRNA 181 (miR-181) is highly expressed in embryonic liver and EpCAM-positive AFP-positive liver cancer subpopulations.[57,58] Also, the tumorigenicity of liver CSCs is significantly suppressed by inhibition of miR-181 which chiefly functions to regulate the differentiation potential of liver CSCs by activating transcription factors such as caudal homeobox gene 2 (CDX2), transcription factor-GATA6 and by negatively regulating the Wnt/β-catenin pathway via nemo-like kinase (NLK).[52,57,58] Moreover, let-7 and Lin28 have been reported to be related to the growth and metastasis of HCC. Lin28 is highly expressed in normal embryonic stem cells and maintains the self-renewal of liver CSCs by inhibiting the combination of let-7 with mature microRNA.[59] Research into microRNA suggests that positive and negative pathways exist for regulating

the differentiation potential of liver CSCs via different microRNAs.[58,59] For negative regulation, degraded let-7 by excessively active Lin28 and c-MYC disequilibrate liver CSCs and all cancer cells that accelerate the growth and metastasis of HCC.[59] Meanwhile, with positive regulation, high expression of EpCAM, which is regarded as a prominent marker of liver CSCs, is mediated by inhibition of TGF-β by downstream transcription factors of miR-18 such as CDX2, GATA6 and NLK.[53,54] EpCAM intracellular domain (EpICD), which is a lysate from EpCAM, enters the nucleus and induces overexpression of cyclin D1, c-MYC and miR-181 after combining with LIM domain protein 2 (FHL2), β-catenin and lymphoid enhancer factor 1 (Lef-1).[58] In addition, Liu's group asserted that TGF-β1 acts through miR-155 to down-regulate TP53INP1 to promote EMT and liver CSC phenotypes.[60] The Wnt/β-catenin signaling pathway that regulates tumor heterogeneity is mainly related to microRNA but how this occurs to sustain the balance of liver CSCs and cancer cells has not been elucidated.

Function of liver CSCs for diagnosis and treatment of HCC

Evidence is accumulating that CSCs are a source of cancer cell and tumor heterogeneity.[6–9] Yang's group reported that liver CSCs from the peripheral circulation had greater tumorigenicity and confirmed that these were significantly positively related to a 2-year relapse after resection.[23] Various liver CSC-related proteins have been reported to be associated with malignance of liver cancer, such as K19 and c-kit.[61] K19 is a keratin on the bile duct cell and c-kit, or CD117, is a stem cell factor receptor. K19 and c-kit are reported to be associated with a range of vascular invasion, tumor diameters, degree of cancer differentiation and APF.[61] Obtaining liver CSCs from the peripheral blood has been made easier with flow cytometry and immunomagnetic beads.[14,19]

Liu's group reported that CD90 has a specificity of 91.9% for HCC and a sensitivity of 48.22% for predicting poor differentiation.[62] In addition, TLR4-positive CSCs were found to be significantly greater in HCC tissues with microvascular invasion and strongly associated with both early recurrence and poor patient survival.[51] Thus, high expression of these markers is significantly associated with poor response to treatment and reduced survival, and measuring and/or monitoring liver CSCs in liver lesions or the peripheral circulation may be an effective tool for surveilling growth, metastasis and relapse of HCC.

Stem cell heterogeneity indicates tolerance for cell death induced by DNA damage due to a survival advantage from their full potential of gene expression[6–9] and this was discovered in liver CSCs that are more tolerant to chemotherapy and radiotherapy and have greater relapse and metastasis after diverse interventions compared to quiescent cancer cells. It has been reported that a specific antibody targeted to EpCAM can more effectively inhibit liver cancer growth that conventional chemotherapy.[31–33,59] In addition, liver CSCs influence sorafenib resistance via Akt/pi3k, WNT, Notch, and IL-6 pathways.[63] Therefore, specific inhibitors targeted to surface markers that directly kill liver CSCs or restrain self-renewal and differentiation may hold promise for tumor treatment.[64–66]

Conclusions

Higher oncogenicity has been reported for hepatic CSC surface markers to suggest a source of tumor heterogeneity but at what stage of differentiation these markers are best monitored is uncertain. Additionally, various liver CSC subpopulations may be more tolerant to radiotherapy or chemotherapy and this may explain the tumor heterogeneity due to hepatic CSC post-translational modifications and epigenetic differences of hierarchical differentiation. Current evidence suggests that measuring peripheral circulating CD90-positive liver CSCs may be useful for early diagnosis and for monitoring potential tumor relapse. Meanwhile, antibodies targeted to liver CSCs with IL-6 or its inhibitors to inhibit self-renewal and survival signaling pathways may hold promise for immunotherapy.

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Insights into novel anticancer applications for apigenin

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Abstract

Flavonoids, naturally occuring derivatives of 2-phenyl-benzo-γ-pyrone, are widespread in plants as coloring substances. Apigenin (4',5,7,-trihydroxyflavone (5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), molecular formula $C_{15}H_{10}O^5$, is a flavonoid present in many fruits and vegetables, primarily in citrus fruits, apples, parsley and celery leaves. It is also found in some medicinal plants, including chamomile flowers, thyme, oregano, peppermint, lemon balm and yarrow, as a 7-O-glycoside with anti-inflammatory and antioxidant activity. In recent years it has attracted a great deal of interest as a bioactive substance reported to have anticancer properties. According to recent literature data, apigenin is able to reduce cancer cell glucose uptake, inhibit remodeling of the extracellular matrix, inhibit cell adhesion molecules that participate in cancer progression and hinder the development of blood vessels needed by growing tumors. It is reported to protect against a wide variety of cancers. The mechanism of anticancer activity is still under investigation and further research is needed.

Key words: flavonoids, apigenin, anticancer agent, chemoprevention

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In recent years there have been many publications revealing the great importance of numerous natural substances as dietary components that have beneficial activity in many diseases of the gastrointestinal tract. Among them are derivatives of phenylchromone, the flavonoids, which exist in nature either as free aglycones or glycosides. Flavonoids have attracted a great deal of scientific and public attention because they are the most common group of plant secondary metabolites present in the human diet.[1] Flavonoids possess a remarkable spectrum of biological and pharmacological activity, including antioxidant, antiallergic, antibacterial, antimutagenic, antiangiogenic and anticancer activity.[2–6] On the molecular level they have been reported to possess properties that modulate different enzyme and receptor activities involved in inflammatory pathologies and cancer.[7,8] Multiple molecular mechanisms of flavonoids, particularly those inhibiting the promotion and progression of cancer cells, have been tested in recent years in the treatment of different types of cancer.[7–9] Some of the tested compounds – quercetin, genistein and flavopiridol (synthetic flavonoid) – have entered the late phase of clinical trials for several oncological diseases.[10–12] Recently apigenin – 4',5,7,-trihydroxyflavone – and its 7-O-glycoside have attracted particular scientific interest as a promising component in cancer therapy.[13] The bioactive flavonoid apigenin has been found in many fruits, vegetables and medicinal herbs, especially in parsley leaves and chamomile flowers. Apigenin occurs in nature along with its glycosides; the apigenin 7-O-glucoside is their basic compound. Another natural derivative of apigenin used in medicine is vitexin (apigenin 8-C-glucoside), which belongs to the subclass C-glycosyl-flavonoids. In vitexin, glucose residue is attached directly to C-8 of apigenin, without an oxygen atom. Vitexin and its 4'-glucoside are used in cardiology to improve coronary blood flow.[1] Apart from its various known pharmacological activities, apigenin has been shown to possess a variety of biological properties, including antioxidant, antiproliferative, chemopreventive and anticancer activities.[2–6,14] Epidemiologic studies suggest that a diet rich in flavones, particularly in apigenin, is related to a decreased risk of certain cancers (of the breast, digestive tract, skin, prostate and some hematological malignancies) and provide strong evidence for a protective role of flavonoids against some malignant neoplasms.[15] As noted above, apigenin is present in dietary sources, mostly as a 7-glucoside, which is hydrolyzed in the gastrointestinal lumen to be absorbed and distributed as apigenin itself. The epithelium of the gastrointestinal tract is thus exposed to higher concentrations of apigenin than other tissues.[1] As Lefort and Blay wrote: "Apigenin is able to reduce cancer cell glucose uptake, inhibit remodeling of the extracellular matrix [and] inhibit cell adhesion molecules that participate in cancer progression."[1]

Modification of the cell cycle

In order to identify the mechanisms of apigenin's activity on the development of the early stages of carcinogenesis and to explain some divergences of reported results, thorough investigations of cell growth and survival, the modulation of angiogenesis and the actions of apigenin on cellular invasion, adhesion and migration were conducted. It has been found that apigenin can inhibit the cell division cycle at different stages. Research has shown that in human tongue squamous carcinoma (SCC-9) and human esophageal cancer (both KYSE-510 and OE33), after exposure to apigenin, the level of cells in the G1 phase (when the cells grow in size and mRNA and high amounts of protein are synthesized) decreased, while the level of cells in the third stage of interphase (the G2 phase) increased.[16] This is associated with the down-regulation of protein cyclin B1 and the up-regulation of p53-inducible gene 3 and protein p21-WAF1. P21-WAF1 is responsible for G1 phase arrest and the G2/M checkpoint, and apigenin in concentration levels higher than 30 μM increases the transcription of this protein. Apigenin can also arrest the cell cycle in the DNA synthetic phase (S-phase). This action was shown in a gastric cancer cell line (SGC-7901).[1]

Apoptosis and angiogenesis

The natural process of apoptosis (programmed cell death) can be enhanced by apigenin in different ways.[17] Besides induction of p21/WAF1 protein (which is also involved in the cell division cycle), apigenin also leads to changes in the phosphorylation of p53 tumor suppressor protein, induction of the nonsteroidal anti-inflammatory drug-activated gene (a growth factor involved in anti-tumorigenesis) and caspase-3 activity.[18,19] Another way in which this flavonoid promotes programmed cell death is through the modification of the Bax/Bcl-2 protein ratio and the release of cytochrome C to the cytoplasm, which binds apoptotic protease activating factor-1.[1] Angiogenesis is a process that provides oxygen and nutrients to a growing tumor through the development of a supportive vascular network. It is made possible by pro-angiogenic molecules, among which vascular endothelial growth factor (VEGF) is the primary regulator. VEGF is rate-limited in pathological angiogenesis.[1] It acts by binding to the VEGF receptor 2 on endothelial cells, finally causing their migration. Research has shown that VEGF receptor expression increases as a tumor becomes more invasive and aggressive.[20,21] Modest exposure to apigenin (up to 20 μM) inhibits VEGF promoter activity, causing impaired VEGF signaling which decreases the capacity of tumor cells to migrate. This action of apigenin has been found in gastrointestinal, breast and prostate cancer cells, as well as in hepatocellular carcinoma.[20,21]

Inhibition of GLUT-1 expression

Cancer cells adapt to their tumor microenvironment to utilize nutritional energy sources better; one way this happens is through overexpression of glucose transporters (GLUT), which helps transport glucose into the cell. Apigenin possesses the ability to downregulate the GLUT-1 transporter, therefore inhibiting glucose transport.[16,22,23]

ECM degradation

There is a group of enzymes that participates in the digestion of components of the extracellular matrix (ECM). This group includes a proteolytic enzyme family – matrix metaloproteinases (MMPs), the main proteolytic enzymes in tumor invasion – which allows cancer cells to degrade the ECM, penetrate the basement membrane and move to other regions. MMPs also regulate cellular adhesion, making it easier for cancer to migrate.[1] Tumor invasion is connected with the tissue inhibitors of matrix metaloproteinases (TIMPs), which regulate the expression and activity of MMPs. Flavonoids, including apigenin, may inhibit cell migration by having a considerable impact on matrix metaloproteinases activity.[1,24]

Impact on cell adhesion molecules

Cell adhesion molecules (CAMs) are proteins that play a significant part in binding cells to other cells or surfaces (such as the extracellular matrix). CAMs include four groups: cadherins, integrins, selectins (calcium-dependent) and the immunoglobulin superfamily (calcium independent). Lost or decreased cadherin expression is observed in some epithelial cancers. This process increases movement and invasion in these cells. Apigenin can heighten the expression of cadherin and in this way can suppress tumor formation.[23,24] The expression of vascular cell adhesion molecules (VCAM) is also modulated by apigenin. Researchers have shown that intraperitoneal injections of this flavonoid decrease the amount of VCAM on endothelial cells of lung capillaries, demonstrating that apigenin can resist tumor metastasis.[25] Integrins are responsible not only for adhesion to the extracellular matrix, but also for cellular shape, motility and gene transcription. The activation of these proteins can induce downstream signaling events, which recruit and trigger kinases, e.g. focal adhesion kinase (FAK).[26] Overexpression of this kinase promotes invasion and is observed in many tumor cells. Apigenin reduces FAK expression and phosphorylation, and also induces its degradation.[27,28] Chemokines – chemotactic cytokines – are small signaling proteins. There are 2 important components that are involved in the metastasis of colon and pancreatic cancer: CXCR4 chemokine receptor type 4 and its ligand, stromal-derived-factor-1 (CXCL12). Activation of the receptor activates molecules essential for chemotaxis, tumor resistance, growth and gene transcription.[29,30] High CXCR4 expression results in the spread and relapse of the tumor, and also a poor prognosis because of its association with proliferation, motility and enhanced VEGF release.[31,32] Another receptor that responds to CXCL12 is C-C chemokine receptor type 7 (CXCR7).[33] The induction of CXCR7 and CXCR4 activating pathways depends on cluster of differentiation 26 (CD26).[34] This enzyme suppresses CXCL12's ability to promote the metastasis and progression of the tumor. Apigenin enhances the expression of CD26 in tumor cells, where it is often lowered.[35]

Other anti-cancer properties of apigenin include scavenging free radicals, inhibiting ornithine decarboxylase (an important factor in tumor growth) and enhancing the concentration of glutathione, which is important for reducing oxidative stress.[1,7,14] Apigenin is well known as an inhibitor of kinase C, mitogen-activated protein kinase (MAPK) and other kinases. Research has shown that a reduction of MAPK leads to the inhibition of cell proliferation in human prostate cancer and apoptosis in anaplastic thyroid cancer.[36] Apigenin can also lower the concentrations of the enzymes aromatase and 17b-hydroxysteroid dehydrogenase. Breast and prostate cancer growth is inhibited by the influence of apigenin on estrogen receptor b-1.[37]

Reducing oxidative DNA damage

The process of carcinogenesis is closely connected to oxidative DNA damage by free radicals, reflected by levels of 8-hydroxy-2 deoxyguanosine (8-OHdG), which results from oxidative modification of guanine. Incorporated into DNA, 8-OHdG leads to point mutation via an adenine and thymine substitution. High accumulations of 8-OHdG in tissues are observed when there is strong DNA damage. Studies have shown that apigenin reduces this damage by protecting cells from oxidative-mediated cellular injury, due to decreased levels of 8-OHdG in H_2O_2-treated cells exposed to apigenin. This flavone is incorporated into the nuclear matrix, where it binds to nucleic acid bases. This has been observed in prostate cancer cells.[38]

Differentiation-induction effect

"Differentiation therapy" is the concept of treating leukemia by forcing the malignant cells to undergo terminal differentiation instead of inducting cell death.[36] It plays an important role in hematological cancer where cell differentiation is inhibited at a particular stage in its maturation. Flavonoid apigenin-7-glucoside can induce acute myeloid leukemia HL-60 cells and chronic myeloid leukemia K562 cells to differentiate the granulocytic

and the erythrocytic lineages, which is possible because apigenin increases the expression of alfa and gamma hemoglobin genes. Studies have shown that this activity may come from a free hydroxyl group at the C-4' position.[39]

Conclusions

Apigenin is a non-toxic dietary flavonoid naturally occurring in plants, vegetables and fruit. It has been known for many years as an antioxidant and anti-inflammatory agent. Recent studies have shown promising new possibilities for apigenin's anticancer activity. It protects and counteracts a wide variety of cancers, with high selectivity for cancer cells as opposed to non-cancerous cells. The mechanism of its action is still under investigation and further research is needed.

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Oral cavity health among cystic fibrosis patients: Literature overview

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Abstract

Cystic fibrosis is a genetic disorder in which the mutation of the Cystis Fibrosis Transmembrane Conductance Regulator (CFTR) gene that codes the protein forming a chloride channel of epithelial cells results in its distorted functioning. The manifestations of the disorder are mainly observed in the respiratory and digestive system. Accumulation of sticky and thick mucus is the dominant clinical symptom; it leads to chronic infections and gradual tissue destruction. Although cystic fibrosis remains incurable, it is currently feasible to extend patients' life expectancy thanks to modern therapy possibilities. As cystic fibrosis is no longer the domain of pediatricians, health care to CF patients needs to be provided by doctors of various specializations. The multidisciplinary team of doctors should include a dentist aware of specific prevention and treatment needs of this group of patients. It results from the fact that in the course of cystic fibrosis it is possible to observe a variety of changes in the oral cavity environment. The study presents dental issues observed in CF patients and reported in literature. Particular attention was paid to dental caries, mineralization disorders of hard dental tissues, gingivitis and the change in the content and properties of saliva; moreover, prevention and treatment options regarding oral cavity health is this group of patients were taken into consideration.

Key words: cystic fibrosis, gastrointestinal disorders, oral disease, genetic markers

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Cystic fibrosis is a genetic inherited disorder in an autosomal recessive manner and caused by mutations within the CFTR protein coding gene (Cystic Fibrosis Transmembrane Conductance Regulator) on the long arm of chromosome 7. It is a systemic disease with different clinical expression. Fully symptomatic cystic fibrosis is characterized by bronchitis, pneumonia, exocrine pancreatic insufficiency (EPI), and a pathognomonic increase in the concentration of Cl^- in sweat. Patients affected by the disease show many symptoms in other organs. In many cases no pancreatic insufficiency is observed. The length and quality of life is usually determined by the changes in the respiratory system. The basic genetic defect leads to the production of thick, sticky mucus and its accumulation, which results in chronic infections and gradual destruction of tissues and organs.

Apart from the changes typical for fully symptomatic cystic fibrosis, numerous changes in the oral cavity may occur as well. This study presents dental problems experienced by cystic fibrosis patients and reported in the literature. Special focus was placed on caries, mineralization disorders of hard tissue, gingivitis, and changes in the content and properties of saliva. Among a multidisciplinary team of doctors that take care of a cystic fibrosis patient, there should be a dentist whose awareness of the specific prevention and treatment needs in this group of patients will make it possible to provide comprehensive treatment.

Introduction

Cystic fibrosis (lat. mucoviscidosis) is the most frequent genetically conditioned total exocrine dysfunction among Caucasians. It is inherited in an autosomal recessive manner and the frequency of its occurrence (1:2000 births) is similar for both sexes.[1–3] Since 2006, due to the high population frequency of the disease, two-stage newborn screening for cystic fibrosis has been conducted in Poland. First, the level of trypsin in the blood is determined, and then a molecular test is conducted. Immunoreactive tripsin (ITR) is measured in a blood sample drawn on a piece of absorbent paper between the 3rd and the 6th day of life. An increased level of ITR may indicate cystic fibrosis; the initial diagnosis is later confirmed with a molecular DNA analysis. With the use of this test, it is possible to establish an early diagnosis and implement professional treatment.

The disease is caused by mutations of the *CFTR* gene, which can be found on the long arm of chromosome 7 (7q31.2), and is responsible for coding protein with the same name. The protein constitutes the apical membrane chloride channel of the epithelioid cells. The structure of the channel consists of several domains which perform specific functions: the endothelial domains set the pathway of chloride ions through the cell membrane, and the regulatory domains are responsible for opening and closing the channel. The process is connected with phosphorylation of protein by cAMP (cyclic adenosine monophosphate) dependent protein kinase.[4] The functioning of the chloride channel is conjugated with the activity of other epithelioid ion channels and transporters. Therefore, it indirectly regulates the flow of carbonate, sodium and potassium ions.[5–7] So far, over 1600 mutations of the *CFTR* gene have been reported. They all result in impairment of the structure and functioning of the chloride channel, which in turn leads to disturbance of the general ion balance of the epithelium.

The most common mutation type diagnosed in 90% of cases is deletion of phenylalanine 508 (ΔF 508 mutation).[7] The ΔF 508 mutation of the *CFTR* gene that causes CF is diagnosed in patients from Northern Europe with a frequency of 75–88%, and in Poland with a frequency of 53–73%. Currently, it is known that apart from classic gene mutations, the phenotype is also determined by polymorphic changes in the *CFTR* gene. Furthermore, there are complex mutations, i.e. 2 mutations, in a single allele.

Organs which contain epithelium accumulate thick and sticky mucus that is very difficult to remove. This, in turn, leads to chronic inflammation and gradual destruction of tissues. Changes in the respiratory and digestive system are dominant in a majority of patients. The mucus accumulated in the area of the bronchial tree stimulates proliferation of pathogens, particularly *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*, which leads to severe chronic infections the complications of which are the most common cause of death.[1,2,4] The fundamental cause of death is development of uneven respiratory insufficiency which may cause death in the first decade of life in the group of untreated cases.[2]

In most cases (85–90%), there are also inflammatory/fibrotic lesions of the pancreas that lead to impairment of its exocrine function.[8] As a result of decreased secretion of the pancreatic juice, digestion and secondary intestinal absorption is disturbed, and this leads to chronic steatorrhea. Its consequences include malnutrition, deficiencies in vitamins (particularly ADEK) and trace minerals, and impaired growth, as well as disturbed bone mineralization in the form of osteopenia and osteoporosis. Some patients, especially those who have suffered from the disease for a long time, also have severe diabetes that is very difficult to control. It results from impaired endocrine function and damage to the pancreatic islets apparatus. Other symptoms connected with the digestive system include meconium ileus which can be observed in the first days of life and may lead to perforation and meconium peritonitis.

Malfunction of the bile ducts in the form of intrahepatic cholestasis leads to inflammation, development of cirrhosis and progressive failure of the organ which in consequence requires a liver transplant. Other complications connected with liver function include increased

occurrence of cholelithiasis which results from abnormal metabolism of bile acid.[1,4] Other conditions related to the digestive system include intensified gastroesophageal reflux, and the so-called equivalents of meconium ileus that afflict older children. Gastroesophageal Reflux Disease (GERD) has a complex pathogenesis, and its intensification may directly impact the progression of bronchopulmonary dysplasia.[9] Increased pain and flatulence may be caused by Distal Intestinal Obstruction Syndrome (DIOS). Partial DIOS is diagnosed when the fecal mass is retained in the ileocecal area. Complete DIOS is characterized by a co-occurrence of bile stained vomiting and/or increased levels of fluid in the small intestine on a plain abdominal X-ray.

Other common pathognomonic symptoms of the disease include rectal prolapse which had constituted a significant diagnostic indicator before screening tests came into use. Moreover, there are reports of an increased risk of gastrointestinal carcinoma.[10] Cystic fibrosis patients may also suffer from infertility which predominantly concerns men (as a result of ejaculatory duct obstruction), as well as from vasculitis and arthritis. Fertility-related dysfunctions among men pose a grave problem among adults suffering from cystic fibrosis, and before screening tests were introduced, they had served as an indication that tests towards CF should be done, and as a result, late diagnosis was established. There are also characteristic changes in the composition of sweat which becomes abnormally salty as a result of increased concentration of chloride ions.[4]

In CF diagnosis, in addition to the aforementioned screening test, a sweat test is used which shows a significantly high concentration of chloride anions in sweat (Cl^- > 60 mmol/L, in newborns > 40 mmol/L), in at least 2 independently done tests. As an alternative to the sweat test, a conductometric test that analyses the electrical conductivity of sweat is done. Should diagnostic difficulties arise (i.e. inconclusive results of the sweat test, lack of two mutations of the *CFTR* gene), the transepithelial nasal potential difference may be measured. The ultimate confirmation of the diagnosis is a molecular analysis and detection of one of the disease specific mutations. Exocrine pancreatic insufficiency can be confirmed by low concentration of fecal elastase-1.

The treatment of CF is interdisciplinary and it mainly concerns the respiratory and digestive system. The therapy of respiratory complications is based on mucolytics, carefully selected antibiotics, aerosol therapy and kinesitherapy.

Gastrointestinal treatment is mainly based on nutrition intervention, supplementation with pancreatic enzymes, administration of vitamins and macro- and microelements the role of which is to prevent malnutrition and disturbed bone mineralization. Therapy with pancreatic enzymes is particularly important. Enzymatic supplementation is necessary when clinical symptoms of exocrine pancreatic insufficiency, i.e. chronic diarrhea, particularly steatorrhea, and small body weight increase, have been observed. The dose of pancreatic enzymes is considered to be proper when it leads to decreased loss of fat with stool (normalization of stool frequency and type), elimination of flatulence and abdominal pain, and continual increase in the body weight.

Although cystic fibrosis remains incurable, progress in the therapy has resulted in significant prolongation of patients' lifespan. Several decades ago, most CF patients died in the first decade of life.[4] Currently, average life expectancy is 37 years; therefore, cystic fibrosis is no longer the sole domain of pediatricians.[1,2] Multidisciplinary medical care helps ensure the best possible comfort of life. This should also include dental treatment and preventive dental care, since this group of patients may demonstrate specific treatment needs.

The aim of the study is to highlight dental problems reported in current literature among patients suffering from cystic fibrosis.

Dental caries

The presence of sugars in the oral cavity which are delivered mainly with food is a necessary condition for the development of dental caries. Carbohydrates are used by cariogenic bacteria, particularly *Streptococcus mutans* and *Lactobacillus acidophilus*, for metabolic processes. As a result of these transformations, the pH of the environment is reduced below the critical values, and the demineralization process of the enamel may begin. The consequence of this process is carious lesions.[7] The most negative effects are observed as a result of even small amounts of carbohydrate substrate.

When dealing with CF patients, special attention needs to be paid to the presence of risk factors related to dental caries. Exocrine pancreatic insufficiency and malabsorption necessitate constant supplementation with pancreatic enzymes, and following a special diet. Daily calorie requirement in the case of CF is 130–150% of the regular requirement for healthy adults.[11] It is met by consuming high-energy meals, usually rich in fat, carbohydrates and protein.[12] It is also recommended to use supplements frequently containing glucose polymers which break down in the oral cavity to monosaccharides. The supplements are typically used in the form of drinks or powders added to dishes.[13] Young children take medication in the form of sweet syrups or suspensions which, if administered frequently and over long periods of time, may cause the development of carious lesions, especially in upper primary teeth.[14] Aerosol medications characterized by acid reaction and containing sugar may also be a potential risk factor in the development of dental caries.[3] Special attention is also paid to overprotective parents who focus on the primary disease and simultaneously

disregard oral health, neglect oral hygiene and allow too frequent consumption of sweets.[13] Despite the risk factors of the development of dental caries observed in the course of CF, reports on the issue presented in the literature are inconclusive.

Laboratory tests have shown drastic development of dental caries and fast destruction of dental crowns among mice with the ΔF 508 mutation after only several weeks of being fed a cariogenic diet. At the same time, a significant increase in the bacterial colony of *Streptococcus mutans* and a decrease in the concentration of hydrocarbonate ions in the saliva have been observed. Impaired function of this main buffering system and a resulting decrease in the pH of the saliva significantly influence the rapidly progressing enamel demineralization process.[7] Certain clinical research seems to confirm an increased risk of dental caries among CF patients. Olejniczak et al. determined a significantly more frequent occurrence of dental caries in the permanent teeth of children aged 6–12 and suffering from cystic fibrosis.[11] However, in a group of patients aged 1–5, there was a reverse tendency. Moreover, there were no significant differences observed in a group of adolescents aged above 13. Similar results were obtained by Dąbrowska et al.[15] Peker et al., in turn, did not report any differences in a group aged 3–12.[3]

Many authors have emphasized the existence of an interesting phenomenon – a less frequent occurrence of dental caries in CF patients in comparison with healthy subjects.[16–23] According to Kinirons's reports, there is a significantly lower level of dental caries in primary teeth, and a similar yet less strongly marked tendency in permanent teeth.[16] Ferrazzano et al. have confirmed a similar, statistically significant dependency in both primary and permanent teeth in children aged 7–12.[20] According to Aps et al., a significantly higher level of dental caries has been reported in the CF of homozygotes in comparison with healthy ones and heterozygotes.[21] When comparing the dental status of children with CF aged 2.5–16.5 with children suffering from other chronic diseases of the respiratory tract, Narang et al. also observed a certain tendency to a less frequent occurrence of dental caries in the cases of cystic fibrosis.[12] Researchers tend to hold the view that the aforementioned phenomenon may result from more frequent and long-term antibiotic therapy, which reduces the titer of cariogenic pathogens in the oral cavity.[18,24] There are reports of a significantly lower level of *Streptococcus mutans* among patients suffering from cystic fibrosis.[22] The dependency between antibiotic therapy and the titer of cariogenic bacteria may be particularly important in small children, among whom *Staphylococcus aureus* is the dominant pathogen of the respiratory system. The infections it causes are treated with penicillin, which is also effective against streptococci that reside in the oral cavity. After the 10th year of life, the microbiological profile changes; infections caused by *Pseudomonas aeruginosa* are dominant and they are treated with tobramycin, which is an aminoglycoside antibiotic and is not effective against cariogenic pathogens. As a consequence, it is believed that the risk of caries development among patients treated with this antibiotic may be higher.[18,19]

When analyzing the risk factors that influence the development of dental caries, the composition and properties of the saliva are also taken into consideration; however, the reports related to this issue are often inconclusive. In the course of cystic fibrosis, salivary secretion may be impaired and it may be caused by damage to the salivary glands, or it may be a side effect of the medications received as part of the treatment. Decreased salivary flow rate, and a simultaneous decrease in its buffering potential responsible for the acid alkaline balance, may lead to the occurrence of dental caries.[15] However, there are also reports of significantly higher average pH values, buffer capacity and higher concentration of calcium and phosphorus ions in the saliva, which have a positive impact on maintaining the acid alkaline balance of the oral cavity environment among patients suffering from CF.[17,22,25] It is thought that the increased capability of the salivary buffering systems may be connected with the supplementation of pancreatic enzymes administered to CF patients.[24]

Disturbed mineralization of hard dental tissues

Lesions in the enamel may appear in the course of chronic, incurable diseases as a symptom of the disturbed mineralization of teeth. It is a long-term process that takes place in the pre-eruptive period and is based on the gradual precipitation of mineral compounds on the organic matrix of tooth buds. In the primary teeth, the process starts before the baby is born. In the case of permanent teeth, the beginning of the mineralization process is set in the following way: molars – in the perinatal period, incisors and cuspids – in the 1st year of life, premolars – between the ages of 2 and 2.5, 2nd molars – in the 3rd year of life, and 3rd molars – in the 8th–10th year of life. Clinical dysfunctions of this process manifest themselves in the form of fuzzy or confined enamel opacities or hypoplasia (areas of thinner enamel or complete lack thereof). Many etiological factors effecting in such lesions are known and they include, for example, other systemic diseases and medications they are treated with.[26]

Molar incisor hypomineralisation (MIH) is a special form of the described abnormality. It is defined as a developmental enamel dysfunction that involves 1 or more molars with frequently coexisting lesions in the incisors. Clinically, enamel opacities of various sizes and colors are observed. The enamel is soft and porous and tends to destroy easily. The etiology of MIH has not been conclusively explained. Apart from a genetic component,

systemic diseases, medications (e.g. antibiotics) and environmental impact factors from early childhood, particularly occurring in the first 3 years of life, are taken into consideration as possible causes of the abnormality.[18]

Research conducted on laboratory animals has shown a connection between disturbances in ion balance related to the defect of the *CFTR* protein and the occurrence of disturbed enamel mineralization.[27–30] Tuggle et al. have observed a change in the coloration and lack of physiological abrasion of incisors in rats devoid of the *CFTR* gene. Premature degeneration of ameloblasts resulting in altered firmness and coloration of the enamel has been observed in mice with CF.[29] The aforementioned data is confirmed in certain clinical reports.[23,31,32] Ferrezzano et al. have observed developmental enamel defects in 56% of the subjects with CF; the same concerned 22% of the healthy subjects.[31] Hypoplasia of the permanent tooth enamel (23% in the CF patients vs 1.5% in the healthy subjects) was the most frequently observed dysfunction. A more frequent occurrence of disturbed mineralization in the case of CF has also been determined by Azevedo et al.; however, the statistical significance only concerned permanent teeth.[32] The most frequently observed dysfunction was opacities. Similar data was obtained by Narang et al., who compared a group of patients suffering from CF with patients afflicted by other diseases of the respiratory system.[11] However, no dependency between cystic fibrosis and molar incisor hypomineralisation has been confirmed.[17]

Gingivitis

In the literature, there are numerous reports indicating a lower risk of gingivitis coexisting with cystic fibrosis.[15,21,23,32,33] Kinirons et al. have observed a significant negative correlation between antibiotic therapy applied for 4 weeks before the examination and both gingivitis and the amount of dental plaque in patients with CF. Olejniczak et al. have determined a statistically lower percentage of patients with gingival bleeding and dental calculus among patients aged 13–18 and 19–29 in comparison with healthy subjects.[33] A similar tendency has been observed among children aged 6–16, yet with no statistical significance. Different data was obtained by Narang et al., who observed a more frequent, yet statistically insignificant, occurrence of dental calculus and gingivitis in CF patients in comparison with patients suffering from other chronic diseases of the respiratory system.[12] Increased calculus accumulation among CF patients has also been reported by Kinirons.[16] This phenomenon may result from changes in the concentration of calcium and phosphorous in the saliva and an increase in its pH, which conduces precipitation of mineral salts and formation of hard deposits.[16,22]

Saliva

In the course of cystic fibrosis, there is a series of significant changes in the composition and properties of saliva which are an expression of salivary gland dysfunction and a side effect of pharmacotherapy. In proper conditions, the primary secretion of salivary glands shows a concentration of sodium and chloride ions which is similar to plasma and interstitial fluid. While the secretion is moving along the excretory ducts, ions are absorbed by chloride (CFTR) and sodium (ENaC) channels.[7,35] As a result, the solution becomes hypotonic. The distorted function of the CFTR channel leads to increased concentrations of chloride, sodium and potassium ions in the saliva. A similar mechanism occurs in sweat glands; due to this fact, it is suggested that saliva may potentially be used for diagnosing cystic fibrosis as an alternative to the sweat test.[34]

Salivary flow rate is an essential parameter that conditions maintaining homeostasis of the oral cavity environment. Fast secretion results in efficient clearing and supplying ingredients that are necessary for proper functioning of the mucosa, periodontium and teeth. Salivary secretion is controlled by the autonomic nervous system. Experiments conducted on animals have shown a diminished response of the salivary glands to adrenergic stimulation in the course of CF.[35] Certain clinical research has confirmed decreased salivary secretion in the case of CF; however, other authors have not determined such a dependence.[36,3] Inconclusive reports also concern changes in the pH values and buffer capacity, which play a significant role in maintaining acid alkaline balance by regulating the demineralization and remineralization processes of hard dental tissues. Kinirons has reported a significant increase in average values of the aforementioned parameters among patients suffering from CF.[17] In other reports, it has been determined that there is a reverse tendency – a significant decrease in pH levels and buffer capacity.[3,34,36]

In the course of cystic fibrosis, changes may also appear in the organic components of saliva. Salivary proteins play various roles in the oral cavity environment. They protect the mucosa against irritative factors, initiate the formation of the dental pellicle that protects the enamel, facilitate the process of mastication and swallowing, and following carbonates and phosphates, constitute the third buffering system that controls the pH. There is a significant increase in protein concentration among patients suffering from CF which has been observed by Aps et al., Modesto et al. and Livnat et al.[21,36,37] Peker et al., in turn, have not confirmed such a connection. Sialic acid, which is a component of glycoproteins, performs a protective role for the tissues of the oral cavity. By covering the oral mucosa, it protects it against dryness, damage and bacterial penetration. Impaired formation of glycoproteins may be one of the causes of bacterial colonization in the respiratory tract among patients

suffering from CF. Modesto et al. have identified a significant decrease in the concentration of total, free and combined sialic acid in the saliva of cystic fibrosis patients in comparison with healthy subjects.[36] According to the authors, the cause of this phenomenon may lie in using mucolytics which, on the one hand, facilitate clearing the mucus, yet on the other hand, may simultaneously impair proper formation of glycoproteins.

In the course of cystic fibrosis, a significant decrease in the enzymatic activity of salivary α-amylase and the peroxidase system has been demonstrated.[36–38] The former enzyme initiates the digestion of starch, and it is also capable of bonding with bacteria and the pellicle. As a result, it may have an impact on the carious process.[38] The latter, i.e. the salivary peroxidase system, is one of the innate immune systems that function in the oral cavity. In the presence of peroxidase and H_2O_2, thiocyanates (SCN^-) that naturally occur in the saliva are oxidized to hypothiocyanites, which demonstrate antibacterial, antiviral and antifungal properties. A decreased activity of this system observed among patients suffering from cystic fibrosis is probably related to impaired secretion of thiocyanates which is dependent on the CFTR channel.[38]

The latest reports also demonstrate a significantly decreased activity of thromboplastin (factor III of the coagulation parameters) in the saliva, which may lead to delayed wound healing and predisposition to intense bleeding resulting from gingival inflammation.[3]

Summary

Cystic fibrosis is a systemic disease that may also have an impact on the functioning of the oral cavity environment. The occurrence of dental caries in the course of cystic fibrosis is a result of many factors demonstrating protective properties, yet at the same time also increasing the risk of the development of the disease. According to the American Academy of Pediatric Dentistry, children suffering from chronic diseases and demonstrating special health care needs, should constitute a high caries risk group.[13] Most reports indicate a lower risk of gingivitis among patients suffering from CF; however, simultaneously, there is a stronger tendency for dental calculus formation. The role of the dentist will then be the regular removal of dental calculus. Changes in the composition and properties of saliva may result in impaired homeostasis of the oral cavity environment. Dysfunctions in the salivary flow rate, its pH and buffer capacity, as well as the impaired protective and antibacterial role of the saliva, increase the risk of inflammation of the mucosa and damage to hard dental tissues. Therefore, it is necessary to schedule regular dental check-ups, recommend the use of remineralizing and antibacterial rinses, and if needed, the use of artificial saliva products. It is also essential to raise the awareness of patients suffering from cystic fibrosis and their caregivers and inform them about potential problems that may occur in the oral cavity, as well as about possible treatment and prevention methods.

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Trace metal ions release from fixed orthodontic appliances and DNA damage in oral mucosa cells by in vivo studies: A literature review

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Abstract

An overview of professional literature referring to the release of metal ions from fixed orthodontic appliances and their influence on oral mucosa in conditions of in vivo are presented, along with a detailed analysis of the exposure of the cells of cheek mucosa epithelium to metal ions. Electronic databases (PubMed, Elsevier, Ebsco) were searched with no language restrictions. The relevant orthodontic journals and reference lists were checked for all eligible studies. A total of 38 scientific articles were retrieved in the initial search. However, only 7 articles met the inclusion criteria. Statistically significant differences in the levels of the amount of nickel ions, cobalt ions and chromium ions were observed in cells of cheek mucosa. The most biocompatible material used in the production of fixed orthodontic appliances is titanium, and the least biocompatible material is steel, which releases the largest amount of nickel and chromium. Metal ions are released from fixed orthodontic appliances only in the first phase of treatment. It is recommended to conduct further, long-term research on a larger number of patients to define the influence of using fixed orthodontic appliances and biological effect they might have on tissues.

Key words: atomic absorption spectrophotometry, cells of cheek mucosa, chromium ions, nickel ions, orthodontic treatment

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Contemporary orthodontics (orthodontic trade companies and orthodontists alike), take utmost care to apply materials that would be close to 100% biocompatible with the tissues of the patients' oral cavity. Elements which constitute the orthodontic fixed appliances (bands, braces, wires) are manufactured from metal alloys which undergo corrosion in the environment of the oral cavity.[1,2] As a result of this process, metal ions (mostly nickel, chromium, cobalt and iron) are released into the human body.[3,4] These metals are well known to be cytotoxic, mutagenic and allergic agents.[5,6] Biocompatibility studies (in vivo, in vitro and usage tests) aim to evaluate the effects of exposure to the metal ions released from orthodontic appliances to the human body.[7–9] In order to achieve this, different biomarkers of exposure, such as saliva, blood, urine, hair, nails and oral mucosa cells, are used, with each of them having their advantages and disadvantages.[10,11] Blood, as an invasive biomarker, is more difficult to collect; moreover, blood and saliva have a window of detection, of approximately 36–72 h. Hair is a well-known biomarker, easy to collect and store in keeping with the established laboratory procedures, which can provide information about chronic exposure.[12–15] Oral mucosa cells are a non-invasive biomarker that is easy to collect. The advantage of this biomarker is that fixed orthodontic appliances are in direct contact with the cells, which can supply information about ion concentration. Thus, oral mucosa cells are the first tissue where a localized corrosion effect takes place.

The aim of this systematic literature review was to present the concentration of metal ions in the cells of the cheek mucosa of orthodontic patients and their influence on DNA damage in in vivo tests.

Material and methods

Search strategy

The following electronic databases were searched by 2 independent researchers (without language restriction): MEDLINE via PubMed (from 1960 to January 2015), Elsevier (from 1980 to January 2015), Ebsco (from 1980 to January 2015). A manual search of the following journals was performed: American Journal of Orthodontics and Dentofacial Orthopedics, Angle Orthodontics, European Journal of Orthodontics (all from 1980 to January 2015). The following key words were applied in this study: 1. fixed orthodontic appliances; 2a. release of metal ions; 2b. nickel; 3a. in vivo; 3b. cells of cheek mucosa; 4. 1 and (2a or 2b); 5. 1 and (3a or 3b); 6. 1 and (2a or 2b) and (3a or 3b); 7. 1 and (2a or 2b) and (3a or 3b) not in vitro. The combination of key words and the results of looking through the databases are presented in Table 1.

All the articles that satisfied the criteria for being included in this paper review have been qualified on the basis of the contents of their abstracts.

Table 1. Key words which became the sought-after phrases in PubMed resources (search strategies)

No.	Word/phrase	Result
1.	fixed orthodontic appliance	2247
2a.	releasing metal ions	62007
2b.	nickel	34033
3a.	in vivo	656627
3b.	cheek mucosa cells	7947
4.	1 and (2a or 2b)	11
5.	1 and (3a or 3b)	6
6.	1 and (2a or 2b) and (3a or 3b)	3
7.	1 and (2a or 2b) and (3a or 3b) not in vitro	3

The following inclusion criteria were used:

1. Type of studies: clinical in vivo studies conducted on patients before, during and after orthodontic treatment with fixed orthodontic appliances.
2. Patients aged between 12 and 35.
3. All participants have permanent dentition, no amalgam fillings or metal restorations, no previous orthodontic treatment, no palatal or lingual appliances welded to the bands, and no extra-oral auxiliary orthodontic appliances. None of the subjects had oral diseases, systemic diseases, illnesses related to genetic damage, diabetes, and allergies to costume jewelry, watches, or any other sources of nickel or chromium. They were not treated with antibiotics or steroids during the study period. None of the subjects were addicted to cigarettes, alcohol or drugs. They did not use alcohol-based mouthwashes.
4. Comparisons: These included the concentration of metal ions in particular groups in the cells of the cheek mucosa before, during and after orthodontic treatment. An assessment of the amount of damaged cells of the cheek mucosa before, during and after orthodontic therapy was carried out as well.

Quality assessment and control of bias

The quality assessment and control of bias were performed using the application of the methodological checklist for prognostic studies developed by the National Institute for Health and Clinical Excellence of the United Kingdom (Fig. 2).[16] During the ranking of selected studies, it was verified that they had very similar scores, which meant: up to 5 "yes" = high; up to 3 "yes" = moderate; 2 or fewer "yes" = low.

Data extraction

The following data was extracted from the selected articles and tabulated by the 2 authors: (1) source,

(2) material and methods, (3) methods of collecting material, (4) metal ions, (5) concentration of metal ions.

Results

During the initial search, 38 articles were deemed potentially relevant to the review; 21 were rejected, including duplicates. Then, the titles and abstracts of 17 articles were assessed, of which 10 were excluded. Only 7 of them fulfilled the inclusion criteria. The primary reasons for rejection are shown in the PRISMA flow diagram (Fig. 1). The accepted articles are presented in Table 2. The material examined contained only the cells of the cheek mucosa epithelium, which was obtained from patients before, during and after orthodontic therapy. The concentrations of metal ions that were sought contained nickel (all articles), chromium (3 out of 5 articles), cobalt (4 out of 5 articles), iron (1 out of 5 articles), molybdenum (1 out of 5 articles) and titanium (1 out of 5 articles). In the studies mentioned above, the following analytical techniques were applied: ICP-MS (3 papers) and AAS with graphite furnace (2 papers, Table 3). The nonparametric test (Kolmogorov-Smirnov) in statistical analysis turns out to have been more popular (3 papers).

Other tests included the parametric Student's t-test (2 papers), nonparametric Wilcoxon test (1 paper), Fisher's exact test (1 paper), Friedman test (1 paper), Levene's test (1 paper) and Tukey's test (1 paper). Nonparametric tests were used more frequently than parametric tests. In Table 3 we present the concentrations of metal ions in the cells of the cheek mucosa. Tables 4 and 5 present research in which the authors define the number of damaged cells of the cheek mucosa.

The information includes materials and methodology as well as conclusions. The research is presented in chronological order.

Faccioni et al. evaluated nickel and cobalt levels in oral mucosa cells.[17] The fixed orthodontic appliances were in the upper and lower jaw (20 brackets, 4–8 bands each). The metal ions were released during the first 4 or 5 months of orthodontic therapy. Epithelial cells of the buccal mucosa were collected by gently brushing the internal part of the right and left cheek with an interdental brush. The cells were immediately prepared for the cell viability and the comet assay. Nickel and cobalt cellular content was quantified by inductively coupled plasma mass spectrometry (ICP-MS). The authors reported an increase in the cobalt and nickel level in oral mucosa cells in the group treated orthodontically, 2.8-fold and 3.4-fold higher, respectively. The potential genotoxic effects, evaluated by alkaline comet assay, indicated that both metals (nickel and cobalt) induced DNA damage. It was found that the presence of nickel and cobalt released from orthodontic appliances induced DNA damage and reduced the cellular viability of mucosa cells.

Amini et al.evaluated nickel, chromium and cobalt levels in oral mucosa cells.[18] For the patients in the experimental group, the average period since the insertion of the appliance to the time of sample collection was 16 months. Mucosa samples were collected by gently brushing the internal part of the right and left buccal mucosa with an interdental brush. The concentration of nickel, chromium and cobalt ions was quantified using atomic absorption spectrophotometry with a graphite furnace. The authors did not find statistically significant differences in chromium ($p = 0.09$) and cobalt ($p = 0.10$) levels between the experimental and control samples ($p < 0.05$). Unlike chromium and cobalt, the average levels of nickel in the experimental and control patient groups were significantly higher (21.74 and 12.26 ng/mL, respectively).

Hafez et al. evaluated the nickel and cobalt levels in oral mucosa cells.[19] The cells were collected before treatment

Table 2. Research which has been included in the review

Source	Material and methods		Method of collecting material	Metal ions	Methods of measurement	Statistics
	test group (age)	control group (age)				
Faccioni et al. (2003)	55 (12–35)	30 (12–33)	interdental brush	Co, Ni	ICP-MS	Student's t-test
Amini et al. (2008)	30 (18.2)	30	interdental brush	Co, Cr, Ni	AAS with a graphite furnace	Student's t-test
Angelieri et al. (2011)	23 (18.5 ±7)	–	moist wooden spatula	metal ions	not given	Friedman test
Natarajan et al. (2011)	20 (14–24)	20	metal spatula	Ni, Cr	ICP-MS	Kolmogorov-Smirnov test, Mann-Whitney U test
Fernandez-Minano et al. (2011)	5 × 3 groups (12–16)	–	interdental brush	Ti, Cr, Mn, Co, Ni, Mo, Fe	ICP-MS	Kolmogorov-Smirnov test, Levene test, Tukey test
Hafez et al. (2011)	28 (20.2 ±4.4)	18	wooden tongue depressor	Ni, Co	AAS with a graphite furnace	Wilcoxon test Fisher test
Heravi et al. (2013)	25 (16.3 ±3.6)	–	metal spatula	metal ions	not given	Kolmogorov-Smirnov test

Table 3. The concentration of metal ions in particular groups in the cells of cheek mucosa ng/mL

Source	Concentration of metal ions							
	Ni		Cr		Co		Fe	
	test	control	test	control	test	control	test	control
Faccioni et al. (2003)	2.521[1]	0.725 ±0.629[1]	–	–	0.568[1]	0.202 ±0.091[1]	–	–
Amini et al. (2008)	21.74 ±11.41[1]	12.26 ±12.9[1]	4.24 ±1.82	3.46 ±1.65	0.84 ±1.06	0.44 ±0.74	–	–
Natarajan et al. (2011)	level of metal ions before debonding							
	4.09 ±3.20	3.86 ±2.17	3.63 ±3.24	2.71 ±1.73	–	–	–	–
	level of metal ions 30 days after debonding							
	3.84 ±1.94	3.48 ±1.55	2.94 ±1.97	2.26 ±1.73	–	–	–	–
Fernandez Minano et al. (2011)	stainless steel alloy							
	0.04 ±0.07	3.44 ±2.79	0.00	0.00	0.00	0.00	2.01 ±0.46	1.95 ±1.29
	titanium alloy							
	0.00	3.44 ±2.79	0.00	0.00	0.00	0.00	1.24 ±0.79	1.95 ±1.29
	nickel-free alloy							
	0.00	3.44 ±2.79	0.34 ±0.29	0.00	0.00	0.00	5.36 ±2.44	1.95 ±1.29
Hafez et al. (2011)	level of metal ions after 3 months fixed appliance placement							
	0.68	0.52	0.41[1]	0.31[1]	–	–	–	–
	level of metal ions after 6 months fixed appliance placement							
	0.78	0.52	0.78[1]	0.31[1]	–	–	–	–

[1] Statistically essential differences between groups; [2] The authors do not give information about the level of metal ions in the tests, but they define the amount of damaged cells in cheek mucosa.

Table 4. The assessment of the amount of damaged cells in cheek mucosa

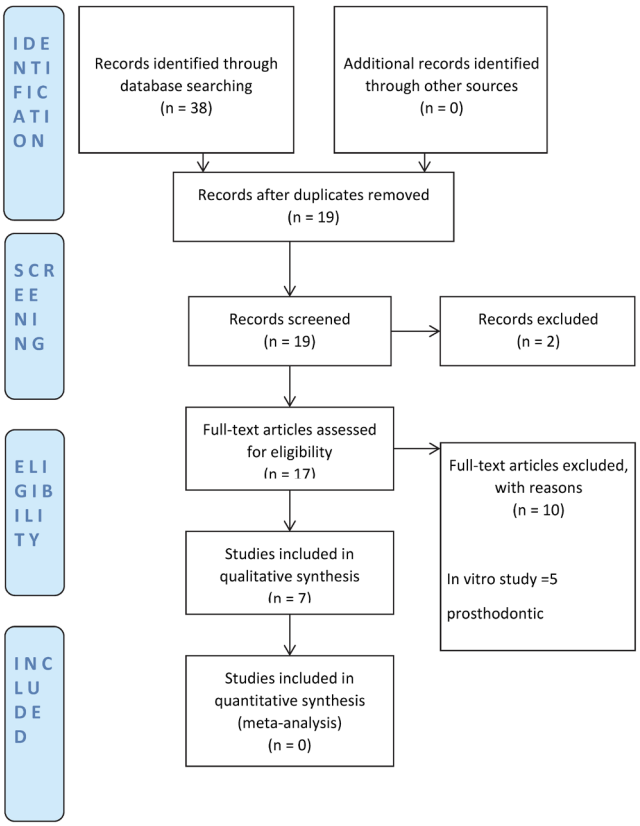
Source	Micronucleated cells			
	before	during	after orthodontic therapy	30 days after debonding
Angelieri et al. (2011)	0.04%	0.07%	0.05%	–
Hafez at al. (2011)	8.1%	6.4%	4.5%	–
Natarajan et al. (2011)	–	–	259 ±233	48 ±49
Fernandez Minano et al. (2011)	30 days after titanium alloy placement			
	25.87 ±3.41	42.6 ±8.93		–
	30 days after stainless steel alloy placement			
	69.35 ±11.68	68.41 ±26.63		–
	30 days after nickel-free alloy placement			
	69.35 ±11.68	68.41 ±26.63		–
Heravi et. al. (2013)	10.6 ±5.7 per 1000 cells	–	9.2 ±6.37 per 1000 cells	–

Table 5. Cytotoxicity and frequency of buccal cells with comet and apoptotic cells in controls (n = 30) and patients (n = 55) with fixed orthodontic appliances for 2–4 years (Faccioni et al., 2003)

Parameters	Controls	Patients	p-value
Comets (100 cells per sample)	11.43 ±6.58	17.62 ±10.08	0.0047
Apoptosis (100 cells per sample)	1.00 ±2.26	3.15 ±4.93	0.021
Viability (%)	73.43 ±12.29	50.40 ±13.55	0.0001

and 3–6 months after the placement of the appliance. Epithelial cells of the buccal mucosa were collected by gently brushing the internal part of the right and left cheeks with a wooden tongue depressor. The cells were immediately prepared for the cell viability and the comet assay. The nickel and cobalt cellular content determinations were carried out with the use of atomic absorption spectrometry with a graphite furnace. The authors reported an increase in nickel from 0.52 to 0.68 and 0.78 ng/mL and chromium from 0.31 to 0.41 and 0.78 ng/mL at 3 and 6 months, respectively. The potential genotoxic effect, evaluated by alkaline comet assay, indicated that both metals induced DNA damage and decreased cellular viability. Compared to the control group, these changes were not evident at 6 months, possibly indicating a tolerance by or repair of the cells and the DNA.

Fig. 1. PRISMA flow diagram



Natarajan et al. also investigated the levels of nickel and chromium in oral mucosa cells.[20] Oral mucosa smears were collected 2 times: at debonding and 30 days after debonding. Mucosa samples were collected by gently brushing the internal part of the right and left buccal mucosa with a metal spatula. The concentration of nickel and chromium ions was quantified using inductively coupled plasma-mass spectrometry. The authors did not find statistically significant differences in chromium and nickel levels between the experimental and control samples. The potential genotoxic effect, evaluated by MN assay, indicated that both metals induced localized genotoxic effects, but did not have a damaging influence on the DNA after fixed appliances had been removed. The nickel and chromium concentrations in the oral mucosal cells were not significantly different from the norm, although the chromium concentration was higher in the experimental group.

Fernandez-Minano et al. evaluated metallic ions (titanium, chromium, manganese, cobalt, nickel, molybdenum and iron) in oral mucosa cells.[21] There was no control group. The authors evaluated metal ions released from 3 alloys (stainless steel, n = 5; titanium, n = 5; nickel-free, n = 5). Samples from the oral mucosa were taken before treatment and 30 days later. Mucosa samples were collected by gently brushing the internal side of the right and left buccal mucosa with an interdental brush. The cells were immediately prepared for the cell viability and the comet assay. The concentrations of titanium, chromium, manganese, cobalt, nickel, molybdenum and iron were quantified using inductively coupled plasma mass spectrometry (ICP-MS). The authors confirmed that among the most biocompatible materials were titanium, then nickel-free and stainless steel, which released the highest amount of ions. Both stainless steel and nickel-free alloys induced more DNA damage in the oral mucosa cells than the titanium alloy.

Angelieri et al. estimated DNA damage (micronucleus) and cellular death in exfoliated buccal mucosa cells from adults after fixed orthodontic therapy.[22] There was no separate control group. The cells were collected by scraping the left and right cheek mucosa with a moist wooden spatula. The cells were then prepared for the cell viability and the micronucleus assay. Before the beginning of orthodontic therapy, the average frequency of micronucleated cells was 0.04%. The conclusion was that the concentration of ions in oral mucosa cells did not increase significantly as a result of orthodontic therapy (before, during and after orthodontic therapy, $p > 0.05$). The authors reported that orthodontic therapy might not be a factor that induces chromosomal damage, nor was it able to promote cytotoxicity.

Heravi et al. also investigated the level of toxic metal ions from orthodontic alloys.[23] There was no separate control group. Mucosa samples were collected by gently brushing the internal side of the right and left buccal mucosa with a metal spatula in a sweeping motion.

Table 6. Methodological checklist for prognostic studies developed by the National Institute for Health and Clinical Excellence from the United Kingdom used to perform the quality assessment and control of bias

	Study sample represents the population of interest with regard to key charakteristics, sufficient to limit potential bias	Loss to follow-up is unrelated to key characteristics sufficient to limit potential bias	Prognostic factor of interest is adequately measured, sufficient to limit potential bias	Outcome of interest is adequately measured, sufficient to limit bias	Important potential confounders are appropriately accounted for, limiting potential bias with respect to the prognostic factor of interest	Statistical analysis is appropriate for the design of the study, limiting potential for the presentation of invalid results
Faccioni et al. (2003)	yes	yes	yes	yes	yes	unclear
Amini et al. (2008)	yes	yes	yes	yes	yes	unclear
Angelieri et al. (2011)	unclear	yes	yes	yes	yes	yes
Natarajan et al. (2011)	yes	yes	yes	yes	yes	yes
Fernandez-Minano (2011)	yes	yes	yes	yes	yes	yes
Hafez et al. (2011)	yes	yes	yes	yes	yes	yes
Heravi et al. (2013)	yes	yes	yes	yes	yes	yes

The oral mucosa cells were collected just before appliance placement and 9 months later. No significant difference was found in the MN count before (10.6 ±5.7 per 1000 cells) and 9 months after therapy (9.2 ±6.37 per 1000 cells, p = 0.336). It was found that the presence of metal ions released from orthodontic appliances did not induce DNA damage and did not reduce the cellular viability of mucosa cells.

Discussion

Typical steel for the production of the parts of fixed orthodontic appliances, like brackets and bands, contains 8–12% nickel.[24] Nickel-titanium arches contain about 47–50% nickel.[25,26] In their in vitro research, Sfondrini et al. proved that the highest amount of chromium is released from new steel brackets (0.52–1.083 μg/g), but less from recycled brackets (0.27–0.38 μg/g).[27]

Some symptoms of allergy to nickel may appear in the oral cavity in patients treated with fixed orthodontic appliances. The symptoms include inflammation of gums and tongue, gingival hypertrophy, erythema multiforme, exfoliation of the lip epithelium and metallic aftertaste in the mouth.[28,29]

Both nickel and chromium may cause infections of the skin and asthma as well as genotoxic and cytotoxic effects in cells and tissues of the body.[29,30]

Additional factors such as the local environment, place of work, working conditions, diet, exaggerated slimming, regular use of alcohol and tobacco as well as individual sensitivity may additionally strengthen the role of nickel and chromium in the etiology of serious general diseases. That is why it seems important to define the number of metal ions that are released daily from fixed orthodontic appliances in both in vitro and in vivo tests.[31–33] The concentrations of metal ions in the cells of the cheek mucosa and the conclusions from the research are different in the analyzed reports. This may result from using differing methods of preparing the material for testing and from applying different analytical methods in the research. Faccioni et al. and Amini et al.report a double or triple increase in the level of nickel and cobalt ions in the cells of cheek mucosa epithelium.[17,18] Statistically significant differences were observed for cobalt (Co)[17], nickel (Ni)[17,18] and chromium (Cr)[19]. In another study, the differences between cobalt and chromium were not statistically significant, although the p-level was low (0.09 and 0.10).[18] In the Fernandez-Minano study, after 30 days of contact with fixed orthodontic appliances, the cheek mucosa cells of the patients increased their concentrations of titanium and manganese.[21] For the patients wearing titanium orthodontic appliances, the manganese content increased and wearers of nickel-free apparatus showed increased concentrations of chromium and iron. Some researchers have failed to find differences in chromium ion concentration.[18] Natarajan et al. have observed that the nickel and chromium ion concentrations in the oral mucosa cells were not significantly different from the norm, although the chromium concentration was higher in the test group. That study has shown that the maximum release of metals occurs within the first 4–5 months.[20]

The different concentrations of metal ions released can be explained by the different experimental methodologies and materials, such as the proportions of the elements in the appliances, the manufacturing of the orthodontic parts

and sometimes also the materials (NiTi wires, SS wires, etc.) and analytical techniques with different lower detection limits. Faccioni et al. used ICP-MS and reported nickel ions of 2.521 ng/mL in the test group and 0.725 ng/mL in the control group, while Amini et al. used AAS with a graphite furnace and reported nickel ions of 21.74 ng/ml in the test group and 12.26 ng/mL in the control group.[17,18] Also, the studies were carried out between the years 2003 (Faccioni et al.) and 2013 (Heravi et al). The sensitivity of the applied analytical techniques has improved significantly in recent years. Additionally, in 3 of the studies, no control group was used.[21–23] Finally, ion release from fixed orthodontic devices in vivo is affected by various factors, such as saliva composition, pH, dietary habits, and microflora.

The assessment of the number of damaged cells of the cheek mucosa was different in the presented studies. Faccioni et al. presented 3 forms damaging the DNA structure (comets, apoptosis and viability), whereas other authors presented one in different units.[17,19–23] The results presented by Angelieri et al. and Heravi et al. showed that the micronucleus frequencies were not significantly different before, during and after orthodontic treatment, and are in contrast with those of Natarajan et al., who found a significantly higher MN count in the test group at the day of debonding as compared to the control group without appliances.[20,22,23] Hafez et al.[19] found that the cytotoxicity and genotoxicity of orthodontic appliances remained in the mouth for 6 months. According to Natarajan et al. and Hafez et al. fixed orthodontic appliances emit metal ions in sufficient quantities to induce a localized genotoxic effect, but these changes were not more evident after a longer time.[19,20] Faccioni et al. have observed the genotoxic damage induced by orthodontic therapy in cheek mucosa cells as assessed by single-cell comet assay in vivo.[17] Their studies showed an increase in the number of comets. This study proved that nickel and cobalt released from fixed orthodontic appliances can induce DNA damage in cheek mucosa cells.

The differences in the results shown in the analyzed articles might result from many causes. One of the most important factors seems to be the length of the time the patient was wearing the fixed appliance, as well as the time of collecting the cells of cheek mucosa epithelium. Another significant factor exerting an influence on the test results might be the method of measuring the damage in DNA (MN assay, comet assay and so on). The remaining differences may stem from a different number of patients in the test group and the control group, lack of control group or the age of the patients.

Conclusions

Stainless steel was the least biocompatible material used in the production of fixed orthodontic appliances since it released the largest amount of nickel and chromium. Titanium, therefore, should be the most preferable material in orthodontic treatment.

The metal ions are only released from fixed orthodontic appliances in the first phase of treatment.

It is recommended that further long-term research on a larger number of patients be conducted to define the influence of fixed orthodontic appliances and their possible biological effect on tissues.

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Coronary artery disease: New Insights into revascularization treatment of diabetic patients

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Abstract

Diabetes mellitus is an independent cardiovascular risk factor, considered an equivalent of coronary artery disease in terms of prognosis. A history of acute coronary syndrome is a strong predictor of another coronary episode, and cardiovascular complications are the leading cause of mortality in diabetic patients. Many patients with coronary artery disease suffer from concomitant diabetes or pre-diabetes. There are 3 strategies of coronary artery disease treatment: conservative management, coronary artery bypass graft (CABG) and percutaneous coronary intervention (PCI). Since drug-eluting stents (DES) were developed, PCI has become one of the most widespread interventional cardiology procedures performed in Europe and worldwide. Among all coronary risk factors, diabetes mellitus remains the most important predictor of unfavorable outcomes of revascularization therapy. This paper reviews the current evidence regarding revascularization in diabetic patients, with particular emphasis on PCI. A systematic analysis of clinical trials of CABG and PCI, especially with DES, was conducted.

Key words: stents, diabetes mellitus, coronary artery bypass graft, percutaneous coronary intervention PCI, drug-eluting stent

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A considerable proportion of individuals diagnosed with coronary artery disease suffer from diabetes mellitus or prediabetes. Previous studies have shown that 6–30% of patients with ST segment elevation myocardial infarction (STEMI) are diagnosed with diabetes mellitus on admission to the hospital. This fraction goes up to 50% if the diagnosis is based on an oral glucose tolerance test.[16]

Numerous studies have demonstrated that patients with diabetes mellitus have a 2–3 times higher risk of mortality due to ischemic heart disease than non-diabetic subjects.[16] Diabetes mellitus may remain undetected for a long period of time, and delayed implementation of treatment, not infrequently resulting from painless onset of myocardial infarction or atypical ailments as a consequence of diabetic neuropathy, is a negative prognostic factor in cardiac patients.

Disorders of carbohydrate metabolism worsen the prognosis in patients with myocardial infarction, especially STEMI. Hyperglycemia correlates with an increase in the levels of inflammatory markers, such as pro-inflammatory interleukins and the von Willebrand factor, higher concentrations of free fatty acids and hyperinsulinemia. This results in further progression of ischemia, higher incidence of life-threatening arrhythmias and an increase in the area of myocardial necrosis. Moreover, hyperglycemia, documented either on admission or during hospitalization, is a strong independent negative prognostic factor in acute coronary syndrome, both in patients with diabetes mellitus and in non-diabetic subjects.

Atherosclerotic lesions in the coronary arteries of diabetic patients

Patients with diabetes mellitus are predisposed for early, systemic and dynamically progressing atherosclerosis, and therefore more frequently require revascularization. Individuals with diabetes mellitus are also at increased risk of multi-vessel coronary artery disease, with a tendency to involvement of the left coronary artery trunk, stenosis of distal arterial segments, the presence of atherosclerotic plaques with vascular thrombosis and calcification. Collateral circulation is usually poorly developed, and no compensatory dilation of arterial lumen is observed in the vicinity of the atherosclerotic plaque. All these disorders predispose patients to worse clinical outcomes of revascularization.[17]

PCI vs CABG

Coronary artery bypass graft (CABG) surgery and percutaneous coronary intervention (PCI) with or without stents constitute alternative types of coronary revascularization. These 2 revascularization methods are among the most widespread interventional cardiology procedures performed in Europe.

CABG remains the treatment of choice in patients with three-vessel disease, stenosis of the left coronary artery trunk and disseminated coronary artery disease, frequently associated with concomitant left ventricular dysfunction. Randomized clinical trials have shown that interventional treatment is associated with longer survival than a conservative approach. Percutaneous intervention is in turn the preferred method of revascularization in most patients with single-vessel disease, as it poses a lower morbidity risk. PCI attenuates the signs of angina and myocardial ischemia in this group of patients.[10]

Patients with diabetes mellitus who have undergone PCI with conventional bare metal stents (BMS) present with both clinical and angiographic evidence of restenosis significantly more often than non-diabetic subjects. Drug-eluting stents (DES) have proven to be safer than metal stents, and their implantation in patients with diabetes mellitus results in lower repeat revascularization rates of the same lesion or vessel than in diabetes-free individuals. Coronary angioplasty is associated with a decrease in intra-hospital mortality and 6-month mortality, from 20 to 7% and from nearly 35 to 12%, respectively.[16]

Randomized trials comparing multi-vessel PCI (with balloon angioplasty or implantation of a metal stent) and CABG have documented the superiority of the latter in patients with diabetes mellitus. CABG resulted in longer survival, a lower incidence of recurrent infarctions and less need for re-intervention in this group of patients.[4]

Review of clinical trials

The FRISC-II Scandinavian randomized trial comparing invasive and non-invasive treatment of unstable coronary artery disease in a group of approximately 2500 patients documented a significant decrease in the incidence of the primary composite end-point of death and recurrent infarction after revascularization.[1,8]

According to data from the Munich Myocardial Infarction Registry, invasive treatment is associated with a significant decrease in the mortality of patients with myocardial infarction and diabetes mellitus.[17] The aim of the study was to compare the long-term outcomes of PCI and pharmacotherapy in patients with stable coronary artery disease and clinical evidence of myocardial ischemia. The study included more than 7500 patients.

Within the framework of the well-known Bypass Angioplasty Revascularization Investigation (BARI) trial assessing long-term survival in 1829 patients with multivessel disease and angina pectoris, a subgroup analysis was conducted to compare the outcomes of PCI and CABG in 353 individuals with diabetes mellitus and multi-vessel disease. The prognosis in patients who

underwent PCI turned out to be worse than in individuals who had surgical treatment; the 7-year survival rates for surgically-treated patients and individuals subjected to PCI were 76.4% and 55.7%, respectively.[1,4]

Notably, similar differences in mortality rates were not documented in the non-randomized BARI registry, which included data from patients in whom the choice of revascularization method was at the physician's discretion.

Another 3 trials comparing the outcomes of CABG and PCI in patients with multi-vessel disease and angina pectoris or clinical evidence of myocardial ischemia, i.e. the Coronary Angioplasty vs Bypass Revascularisation Investigation (CABRI), the Emory Angioplasty vs Surgery Trial (EAST) and the Randomised Intervention Treatment of Angina (RITA-1) confirmed the results of the BARI trial. However, it should be emphasized that all these trials were conducted prior to the implementation of stenting in the PCI protocol (1988–1990), i.e. in the era of balloon angioplasty.[13]

The BARI 2D trial, a large international randomized clinical trial including 2368 patients with a history of diabetes mellitus (10.4 years on average) compared the outcomes of intensive pharmacotherapy and the results of revascularization treatment (PCI or CABG) combined with pharmacotherapy in diabetic subjects with stable coronary artery disease. During a 5-year follow-up, all-cause and infarction-related mortality was significantly lower in persons who had undergone CABG than in individuals who received solely the optimal conservative treatment. However, no significant differences in the incidence of these endpoints were found when individuals who received only pharmacotherapy were compared with those subjected to both PCI and the medical treatment.[2,3]

The results of the Angina With Extreme Serious Operative Mortality Evaluation (AWESOME) trial seem to be inconsistent with the findings of the BARI trial mentioned above. The AWESOME study included solely high-risk patients, i.e. individuals with unstable angina and high surgical risk (after cardiac surgery, with a history of myocardial infarct within the previous 7 days, LVEF < 35%, age > 70 years or subjected to intra-aortic balloon counterpulsation). One third of the patients had concomitant diabetes. The 5-year survival rates for individuals who had undergone CABG or PCI were 34% and 26%, respectively.[1]

The 5-year multicenter SYNergy between percutaneous coronary intervention with TAXus and cardiac surgery (SYNTAX) trial included 1800 patients with left main coronary artery (LMCA) disease or multi-vessel disease, randomized to CABG or PCI with DES. A subgroup analysis was conducted within the framework of this study to determine the effect of concomitant diabetes mellitus on the outcomes of PCI and CABG. The 1-year incidence of serious adverse cardio-cerebral vascular events in individuals with concomitant diabetes mellitus who underwent PCI with implantation of a paclitaxel-eluting stent was twice as high as in diabetic patients subjected to CABG; this difference resulted mostly from a need for repeat revascularization in the former group.[3]

The Arterial Revascularization Therapies Study 2 (ARTS 2) registry of individuals with multi-vessel disease included data from patients with diabetes mellitus who underwent PCI with implantation of a sirolimus-eluting stent (SES), and the ARTS 1 cohort study analyzed diabetics who underwent CABG or PCI with BMS stents. A comparative analysis of these 2 studies demonstrated that the 1-year incidence of adverse events (all-cause mortality, cerebrovascular events, myocardial infarction, repeat revascularization) was markedly lower in the patients subjected to PCI with SES implantation than in the individuals from the CABG group.[5]

Altogether, the evidence from the clinical trials outlined above suggests that patients subjected to various types of revascularization treatment likely do not differ in terms of survival rates. However, patients with concomitant diabetes mellitus more frequently require repeat revascularization and PCI, and restenosis seems to be a significant clinical problem primarily in individuals who undergo coronary angioplasty with BMS implantation.

PCI with stents

The implementation of drug-coated stents in clinical practice was reflected by better outcomes of percutaneous revascularization in patients with diabetes mellitus. A meta-analysis comparing the effects of drug-eluting stents (DES) and conventional bare metal stents (BMS) in subgroups of diabetic patients participating in a few clinical trials revealed that the use of DES was reflected by an 80% decrease in the relative risk of in-stent restenosis during the first year after the procedure. However, if dual antiplatelet therapy lasted less than 6 months, the mortality rates of DES-implanted patients were significantly higher than in individuals treated with BMS. In contrast, the 2 groups did not differ in terms of mortality rates and the incidence of a composite end-point (death and infarction) if dual antiplatelet therapy was continued longer than 6 months. Moreover, regardless of the duration of antiplatelet therapy, the repeat revascularization rate for the same vessel turned out to be markedly lower after DES implantation than following BMS implantation.[19]

Interestingly, the type of antidiabetic treatment also seems to affect the outcome of revascularization. A large clinical trial comparing the outcomes of everolimus- (EES) and paclitaxel-eluting stent (PES) implantation showed that patients who did not receive insulin were at lower risk of repeat revascularization of the same ischemic lesion after EES implantation; in contrast, the risk for repeat revascularization in individuals receiving insulin therapy was lower after PES implantation.[11]

These findings confirm that antidiabetic treatment, especially insulin therapy, may significantly affect clinical outcomes in patients subjected to DES implantation.

Drug-eluting stents

Drug-eluting stents are particularly useful in coronary angioplasty in patients with diabetes mellitus. However, although their use is associated with a lower incidence of post-revascularization restenosis, it also results in a greater risk of acute in-stent thrombosis. DES implantation in patients with diabetes mellitus is associated with better angiographic and clinical outcomes than in the case of BMS. Patients treated with DES less frequently present with restenosis or recurrent myocardial infarction, and less frequently require repeat angioplasty.[4]

Review of further clinical trials

The randomized multicenter trial to assess the use of the cypher sirolimus-eluting coronary stent in acute myocardial infarction treated with balloon angioplasty (TYPHOON) analyzed the safety and efficacy of a sirolimus-eluting stent in 712 patients with acute STEMI, treated with primary PCI with SES or BMS. The study showed that implantation of SES was associated with nearly twice as low 1-year incidence of a composite end-point (death, recurrent infarction, need for revascularization) as in the case of BMS.[20]

The Coronary Artery Revascularization in Diabetes (CARDia) study was the only clinical trial designed to compare the outcomes of CABG and PCI with BMS or DES implantation in patients with diabetes mellitus and symptomatic multi-vessel disease. The study included a total of 510 patients. The overall 1-year incidence of death, infarction and stroke in the CABG and PCI groups were 10.5% and 13% respectively, and the repeat revascularization rate amounted to 2.0% and 11.8%, respectively.[3]

Data from the New York revascularization registry also suggest that the outcomes in diabetic patients who undergo CABG are better than in those subjected to PCI with DES.[2]

The sirolimus-eluting vs paclitaxel-eluting stents for coronary revascularization SIRTAX trial was followed up 5 years later by the SIRTAX-LATE study, both of which involved a group of 1012 randomized patients with coronary artery disease and compared the outcomes of sirolimus- (SES) and paclitaxel-eluting stent (PES) implantation. The incidence of serious cardiac events in patients with diabetes mellitus turned out to be twice as high as among non-diabetics. During the first 5 years after the implantation of the stent, serious coronary events were documented in every 3rd patient with diabetes mellitus and in only 15% of non-diabetic subjects. Some evidence suggested that second-generation (SES) stents are better than first-generation (PES) stents, and the SIRTAX-LATE study showed that SES are superior to PES in patients with diabetes mellitus.[19]

However, neither the Clinical evaluation of the XIENCE V everolimus-eluting coronary stent system in the treatment of patients with de novo native coronary artery lesion (SPIRIT), a large meta-analysis of everolimus-eluting stents (EES), nor the COMPARE trial of everolimus-eluting stents and paclitaxel-eluting stents for coronary revascularization in daily practice, comparing the outcomes of everolimus- and paclitaxel-eluting stent implantation, documented any significant differences in the efficacy of the first-generation (PES) and second-generation devices (EES).[19]

The Randomized Evaluation of Sirolimus-eluting vs Everolimus-eluting stent Trial (RESET) was a Japanese prospective multicenter study study comparing the outcomes of sirolimus- and everolimus-eluting stent implantation; it included a total of 3197 patients, among whom 45% had concomitant diabetes mellitus. Interestingly, everolimus-eluting stents turned out to be superior to sirolimus-eluting devices in individuals with insulin-dependent diabetes.[11,19]

An American observational prospective trial, RESOLUTE, including 1402 patients, among them 34.4% with diabetes mellitus, compared 2 types of second-generation stents (an everolimus-eluting stent and Resolute, a zotarolimus-eluting stent); the study did not find any significant differences between the 2 devices. Irrespective of the type of implanted stent, the incidence of serious adverse cardiac events was very low, and the incidence of in-stent thrombosis did not exceed 1% per year in either group.[19]

The new generation of biodegradable stents

Recently, the use of a novel type of device – biopolymer-coated stents – is increasingly being reported. Preliminary evidence suggests that these devices may pose a lower risk of late in-stent thrombosis than conventional DES. DES implantation results in chronic inflammation of the arterial wall, induced by the durable polymer coating of the stent; this eventually leads to thrombosis. In contrast, the surface of the new biodegradable stents, which is exposed after the release of an antiproliferative agent, resembles the surface of conventional bare metal stents; this results in attenuation of the inflammatory process. In 2012, 3 randomized trials comparing the outcomes of treatment with biodegradable stents and sirolimus-eluting DES (ISAR-TEST 3, ISAR-TEST 4 and LEADERS) were analyzed. The meta-analysis, including data from a total of 4062 patients, showed that compared to SES, biodegradable stents have better clinical efficacy and a better safety profile during a 4-year follow-up. Moreover, the use of biodegradable stents was associated with

lower incidence of late thrombosis. However, it is unclear if the incidence of thrombosis was lower when biodegradable stents were compared with second-generation drug-eluting stents; consequently, no definite conclusions on the superiority of the former devices should be formulated until a comparative analysis of the biodegradable stents with second-generation DES has been conducted.[12]

Conclusions

Diabetes mellitus is an independent cardiovascular risk factor, and cardiovascular complications constitute the leading cause of mortality in diabetic patients. Myocardial revascularization within 14 days of infarction, either STEMI or non-STEMI, results in a 53% and 64% decrease in 1-year mortality of non-diabetic subjects and diabetic patients, respectively.[1] This suggests that individuals with diabetes mellitus may benefit more from revascularization than patients without this condition.

Many studies have demonstrated that CABG is superior to PCI, resulting in longer survival, lower incidence of recurrent infarctions and less need for repeat intervention. Nevertheless, CABG remains the treatment of choice solely in diabetic patients with three-vessel disease, stenosis of the left coronary artery trunk and disseminated coronary artery disease, frequently associated with concomitant left ventricular dysfunction. Consequently, most individuals with diabetes mellitus are subjected to PCI. Diabetic patients are at increased risk of serious cardiovascular events after PCI. Compared to bare metal stents, the use of drug-eluting stents is associated with longer cardiovascular event-free survival and less need for repeat revascularization due to ischemia. Although several large clinical trials have shown that everolimus-eluting stents (EES) are generally safer and more efficient than paclitaxel-eluting stents (PES), this difference is not as evident in the case of diabetic patients. Thus, further research is needed to develop more efficient treatment options for high-risk patients with diabetes mellitus.

Given a choice between cardiac surgical treatment and PCI, diabetic patients are likely to prefer the latter; PCI is less invasive, raises fewer concerns, results in faster improvement of quality of life and a faster return to work, and does not require any specific form of rehabilitation. All this justifies further research on stent improvement. Perhaps biodegradable stents will constitute a solution for diabetic patients with coronary artery disease, and perhaps their use will result in lower rates of restenosis and fewer repeat revascularizations in this group.

Diabetes mellitus is becoming a pandemic and a constant increase in the incidence of this condition is expected. Consequently, awareness should also increase. Progress in research should improve the decision-making process and result in evidence-based optimization of revascularization strategies.

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