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Mieczyslaw Pokorski
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Advancements in Managing Intracerebral Hemorrhage: Transition from Nihilism to Optimism

Sunil Munakomi and Amit Agrawal

Abstract

There have been significant advancements in the management of intracerebral hemorrhage (ICH) stemming from new knowledge on its pathogenesis. Major clinical trials, such as Surgical Trial in Lobar Intracerebral Hemorrhage (STICH I and II), have shown only a small, albeit clinically relevant, advantage of surgical interventions in specific subsets of patients suffering from ICH. Currently, the aim is to use a minimally invasive and safe trajectory in removing significant brain hematomas with the aid of neuro-endoscopy or precise guidance through neuro-navigation, thereby avoiding a collateral damage to the surrounding normal brain tissue. A fundamental rational to such approach is to safely remove hematoma, preventing the ongoing mass effect resulting in brain herniation, and to minimize deleterious effects of iron released from hematoma to brain cells. The clot lysis process is facilitated with the adjunctive use of recombinant tissue plasminogen activator and sonolysis. Revised recommendations for the management of ICH focus on a holistic approach, with special emphasis on early patient mobilization and graded rehabilitative process. There has been a paradigm shift in the management algorithm, putting emphasis on early and safe removal of brain hematoma and then focusing on the improvement of patients' quality of life. We have made significant progress in transition from nihilism toward optimism, based on evidence-based management of such a severe global health scourge as intracranial hemorrhage.

Keywords

Brain hematoma · Brain herniation · Clinical management · Clot lysis · Intracerebral hemorrhage · Minimally invasive surgery

1 Introduction

Intracerebral hemorrhage (ICH) accounts for the most disabling form of stroke (Qureshi et al. 2001). Primary ICH is a sequela to uncontrolled hypertension or amyloid angiopathy (Martini et al. 2012). Secondary ICH, on the other hand, results either from congenital or acquired underlying etiologies such as vascular malformations, tumors, anticoagulation therapy, or cerebral venous thrombosis (Fan et al. 2012). With recent advancements in neurosurgical armamentarium as well as critical care management, the outcomes in ICH seem much more optimistic than in the

S. Munakomi (✉)
Department of Neurosurgery, Nobel Medical College and Teaching Hospital, Biratnagar, Nepal
e-mail: mgmt@nobelmedicalcollege.com.np

A. Agrawal
Department of Neurosurgery, Narayana Medical College, Nellore, Andra Pradesh, India

past times. Herein, we review our present understanding and the management algorithm in managing patients with hemorrhagic strokes.

2 Pathogenesis of the Primary Impact of Intracerebral Hematoma: Implications of Hematoma Removal

When the blood exsanguinates and intracerebral hematoma is formed, the heme oxygenase enzyme cleaves heme, thereby releasing iron that contributes to cellular toxicity (Morioka and Orito 2017). The resultant mass effect of a hematoma encompasses mechanical damage to the surrounding white matter zone. White matter injury is further escalated with secondary cascades of inflammatory, oxidative, and excitotoxic mediators, exemplified by nuclear factor kappa B (NF-κB), matrix metallopeptidase 9 (MMP-9), and glutamate, leading to demyelination and axonal disintegration (Tao et al. 2017). ICH size is a powerful determinant of subsequent outcome (Broderick et al. 1993). Poor outcomes are invariably seen among patients of advanced age, hematoma volume of more than 60 mL, and a score of 6 or fewer points on the Glasgow coma scale (GCS) (Pai et al. 2007). Increased intracranial pressure diminishes cerebral perfusion, which is a major factor causing brain ischemia (Zheng et al. 2016). Increased intracranial pressure may also lead to midline brain herniation, one of the main factors contributing to the death in patients with ICH (Arboix et al. 2002). Such complications of ICH make the timely surgical evacuation of hematoma of paramount importance. Such a surgical procedure is conducive to restoring normal brain perfusion as it would mitigate the increase in intracranial pressure and thus also in brain edema (Zhang et al. 2014).

The International Surgical Trial in Intracerebral Hemorrhage (ISTICH) and the subsequent STICH II study have demonstrated a small survival advantage, with no functional improvement, in patients having just superficial lobar hemorrhages (Mendelow et al. 2005; Mendelow et al. 2013). A timely hematoma evacuation is appealing since it minimizes secondary edema, reverses mass effect with a resultant midline shift and herniation, and reduces toxic neuronal effects of iron coming from erythrocyte lysis (Tao et al. 2017). The acute brain damage from ICH, the trauma associated with surgical access, and a risk of rebleeding confer bad outcome in many patients (Morgenstern et al. 2001; Teernstra et al. 2006). In putaminal or lobar ICH, neurological condition (either hemiplegic or hemiparetic) of a patient should be taken into consideration since removal of hematoma can significantly improve hemiparesis owing to the compression of the internal capsule (Reichart and Frank 2011). Hematoma evacuation could be lifesaving when secondary neurological deterioration occurs or in the case of a large volume ICH with herniation syndrome, especially in young persons and in temporal lobe ICH (Dastur and Yu 2017). In other cases of ICH, the benefit stemming from hematoma evacuation seems somehow neutralized by the inadvertent surgical trauma while accessing hematoma (Zuccarello et al. 1999). That has spurred a search for novel techniques of hematoma evacuation devoid of wider brain exposure and with minimal tissue retraction and manipulation (Dey et al. 2014; Abdu et al. 2012).

A protocol of minimally invasive surgery and recombinant tissue plasminogen activator (rt-PA) (MISTIE) for ICH evacuation has evaluated clinical outcomes following hematoma evacuation taking the shortest path through anterior, posterior, or the longitudinal trajectories (Morgan et al. 2008). Such minimally invasive techniques along with hemostatic agents have helped reduce the stay in intensive care units from 11 to 6 days compared to the craniotomy counterpart (Wang et al. 2015a, b). Precision-guided minimally invasive aspiration or evacuation of ICH, presented in the MISTIE II study, has shown a significant association of hematoma removal with edema reduction in a surgical group of 79 patients (Mould et al. 2013). Such approach has also led to a significant neurological improvement, although unfortunately with no real mortality benefits (Wang et al. 2009). The MISTIE III trial with stereotactic computed tomography

(CT)-guided endoscopic surgery arm via trans-sylvian and trans-insular approach yields better results as it spares cortical function (Przybylowski et al. 2015; Spiotta et al. 2015). Preliminary trials of clot lysis, evaluating accelerated resolution of intraventricular hemorrhage (CLEAR III), have failed to show outcome benefits. The influence of ultrasound, enhancing the effectiveness of thrombolytic clot dissolution, has been studied in vitro. The results have shown faster lysis of intracerebral or intraventricular hemorrhage in patients treated with sonolysis combined with the use of rt-PA versus rt-PA alone (Newell et al. 2011). Stereotactic underwater blood aspiration (sCUBa) in ICH, with composite dry and wet field techniques, has been developed to counteract the limitations of obstructed visualization and poor control of bleeders, which also pertains to minimally invasive approaches (Kellner et al. 2018). Ultra-early surgery within 4 h of onset of ICH episode has led to a higher perioperative rebleeding in comparison to delayed surgery within 12 h from onset (Morgenstern et al. 2001). Though minimally invasive and neuroendoscopic surgery could remove hematoma, craniotomy still remains the most effective treatment strategy to lower intracranial pressure in patients with herniation syndrome following ICH (Cai et al. 2017). In ICH, owing to a high risk of early brainstem compression, surgery is indicated when hematoma diameter exceeds 3 cm or when there is evidence of brainstem compression or hydrocephalus (Becker et al. 1999). In such cases, placement of an external ventricular drain alone is insufficient as it leads to transtentorial upward brainstem herniation and risk for intensified neurological decline (Chan and Hemphill 2014).

3 Secondary Progression and Management of Intracranial Hemorrhage (ICH)

It has been seen that more than 20% of patients deteriorate within a few hours of the onset of ICH owing to hematoma expansion (Rodriguez-Luna et al. 2013). Early deterioration is primarily governed by the hematoma size, its expansion, and the associated intraventricular hemorrhage. Large edema volumes and fever have been found to be determinants of early deterioration within 24–72 h. Medical complications and diminished functional brain reserve determine delayed health deterioration within weeks following ICH (Lord et al. 2015). Worsening perihematoma edema associates with negative outcome (Murthy et al. 2015; Yang et al. 2015). Neurological deficits developing during the first week after ICH have been associated with 1 year mortality of 60.5%, compared to 9.2% among the patients who remain stable (Ovesen et al. 2015).

There are multiple factors that connote acute hypertensive response seen after strokes. These factors entail the underlying uncontrolled hypertension, a secondary effect of the Cushing-Kocher response, and a catecholamine surge (Alqadri et al. 2013; Arboix et al. 2004; Cheung and Hachinski 2004; Qureshi et al. 2000). CT angiograms help identify a "spot sign" that is presage of hematoma expansion. The angiograms also rule out any other underlying vascular abnormalities behind the bleed (Fan et al. 2012). Recently, a "leakage sign" and a "blend sign" have been found superior to "spot sign" in predicting hematoma expansion and, consequently, neurological deterioration (Sporns et al. 2017). Magnetic resonance imaging (MRI) is a useful tool to better stratify the underlying etiologies such as amyloid angiopathy or tumors. However, transportation of these critically ill patients to the magnetic resonance suite and the time taken for relevant image acquisition are the major hindrance limiting a wider application of MRI during the crucial initial phase of patients' stabilization (Smith et al. 2005). The ICH score has been a reliable tool in presaging mortality within 30 days of the insult (Jamora et al. 2003). Recently, the National Institute of Health Stroke Severity score (NIHSS) has been used to reliably predict hospital mortality after ICH, whereas functional outcome score (FOS) best assesses the functional patients' outcome (Satopää et al. 2017).

In chronically hypertensive patients, since there is a rightward shift in the autoregulatory curve normally set between 50 and 150 mmHg, there is an evident risk of ischemic injury following a sudden decrease in the intracranial pressure (Mariano et al. 2012). MRI performed in diffusion and perfusion studies has depicted hypoperfusion with a diminished diffusion coefficient in the penumbra zone subsequent to aggressive blood pressure management (Kidwell et al. 2001). However, positron emission tomography (PET) has demonstrated that oxygen extraction fraction (OEF) is matched to a reduction in metabolic demand despite hypoperfusion present in penumbra zones (Zazulia et al. 2001). Similar studies using CT perfusion modalities performed in the intracerebral hemorrhage acutely decreasing blood pressure trial (ICH-ADAPT) have verified the presence of a preserved autoregulatory mechanism with no mismatch seen between the OEF and the cerebral metabolic rate of oxygen ($CMRO_2$) in these zones despite aggressive antihypertensive therapy (Butcher et al. 2013; Kate et al. 2014; Powers et al. 2001).

Recent meta-analyses have not substantiated any significance of intensive blood pressure lowering treatment in either preventing hematoma progression or reducing dependency or mortality (Boulouis et al. 2017; Lattanzi et al. 2017). The INTERACT-2 trial failed to demonstrate any adverse events following the aggressive lowering of systolic blood pressure below 140 mmHg compared to that below the 180 mmHg level (Anderson et al. 2013). However, the antihypertensive treatment of acute cerebral hemorrhage (ATACH) II trial with a lower cutoff limit of systolic blood pressure than that in the INTERACT-2 trial, about 130 mmHg versus 150 mmHg, respectively, has demonstrated a higher incidence of adverse renal events (Qureshi et al. 2016). To limit the potential occurrence of such adverse effects, some studies suggest the targeting of systolic BP at 130 mmHg (Gregory and Daniel 2018). Intravenous calcium channel blockers and β-blockers, owing to their short half-life and easy titratability, are recommended for blood pressure management in ICH (Calhoun et al. 2008).

The 24-hour blood pressure variability appears to play a critical role in the neurological sequelae of hemorrhagic stroke (Zhang et al. 2018). Persistent systolic blood pressure control, with minimum variability, is the cornerstone in reinforcing the beneficial effect of intensive antihypertensive treatment (Jeon et al. 2018). Intravenous administration of hypertonic saline is more effective in managing enhanced intracranial pressure compared to mannitol therapy (Kamel et al. 2011). Mannitol has failed to modify outcome among patients enrolled in the INTERACT-2 study (Wang et al. 2015a, b).

4 Holistic Approach in Patient Care

A prophylactic phenytoin use has shown to adversely affect outcomes in patients with ICH (Messé et al. 2009; Naidech et al. 2009). Therefore, the use of antiepileptics should be restricted only to patients with clinical seizures and those demonstrating abnormal EEG recordings (Becker et al. 1999). Likewise, coagulopathies, hyperglycemia, and sustained fever are also associated with worse outcomes (Lord et al. 2014; Huhtakangas et al. 2011; Passero et al. 2003). Mechanical, thromboembolic prophylaxis, preferably with intermittent pneumatic compression devices, should be initiated from the time of admission of ICH patients. Prophylactic subcutaneous unfractionated heparin or lower molecular weight heparin (LMWH) should be started in stable brain hematomas within 48 h of admission (Nyquist et al. 2016). Preventing muscle atrophy with appropriate physiotherapy is another factor that plays a major role in neurological improvement (Munakomi 2018). All these factors have been thoroughly emphasized in the updated guidelines for managing patients with hemorrhagic strokes (Tables 1 and 2) (Hemphill et al. 2015).

Table 1 Revised guidelines for management of patients with intracranial hemorrhage

Vitamin K in the cases of elevated international normalized ratio (INR) due to vitamin K antagonists
Intermittent pneumatic compression beginning on the day of admission
Prothrombin complex concentrate considered over fresh frozen plasma
Platelet transfusion in antiplatelet history uncertain
Recombinant factor VIIa (r-FVIIa) not recommended for vitamin K antagonist reversal
Graduated compression stockings not beneficial to reduce deep vein thrombosis or to improve outcome
Lowering of blood pressure to 140 mmHg effective in improving functional outcome
Lowering of blood pressure to 140 mmHg safe
Expert care in intensive care unit
Glucose monitoring
Electroencephalography (EEG) monitoring in patients with depressed mental status out of proportion to the degree of intracranial pressure, assessed on Glasgow coma scale (GCS) of <8, herniation, and intraventricular extension of hemorrhage with hydrocephalus; maintain cerebral perfusion pressure at 50–70 mmHg
Efficacy of recombinant tissue plasminogen activator (rt-PA) uncertain
Usefulness of surgery not well established
Minimally invasive clot evacuation role uncertain
Decompressive hemicraniectomy might reduce mortality in patient with coma, midline shift, and elevated ICP
Postponement of "do not resuscitate" and aggressive care for 48 h
Lobar location, older age, micro-bleeds, anticoagulation, apolipoprotein E2 and E4 allele risk factors
Lifestyle modifications, including treatment of obstructive sleep apnea syndrome probably beneficial
Multidisciplinary rehabilitation
Blood pressure control in all cases

Table 2 Latest recommendations for management of patients with intracranial hemorrhage

Protamine sulfate to reverse heparin
Systemic anticoagulation or inferior vena cava (IVC) filter in intracranial hemorrhage with symptomatic deep vein thrombosis or pulmonary embolisms
Activated charcoal if less than 2 h for novel oral anticoagulants (NOACs), dialysis for dabigatran
Treating fever
Screening for dysphagia prior to oral intake
Screening for myocardial infarct
No administration of steroids
Efficacy of endoscopic treatment uncertain
Early hematoma evacuation not beneficial
Evacuation in deteriorating patient lifesaving measure
Blood pressure target of <130/80 mmHg
Blood pressure control immediately
Aspirin monotherapy can be restarted in a few days following intracranial hemorrhage
Usefulness of newer NOACs to decrease recurrence uncertain
Optimal time to resume oral anticoagulation uncertain; in mechanical heart valves the laps of at least 4 weeks

5 Future Perspectives in Management of Intracranial Hemorrhage (ICH)

A collaborative future research is likely to yield therapies for this devastating form of brain injury (NINDS ICH Workshop Participants 2005). Multimodal neuro-monitoring with transcranial Doppler (TCD) and quantitative electroencephalography (EEG) can provide better clinical management of ICH patients (Chen et al. 2018). Focusing on the neurological outcome alone after surgery seems insufficient in medical management of ICH. We now attempt to sway away from the nihilistic views concerning the

inescapable mortal sequelae of ICH. A multidisciplinary holistically oriented approach is essential for achieving best patient outcome (Godoy et al. 2015). The nutshell of a current perspective in the surgical management of ICH is that "we are now moving forward rather than in circles". However, the main bias in such approach may be the lack of "equipoise" during randomization of these cohorts into surgical and nonsurgical management and taking determinants of outcome in terms of only Glasgow outcome score (GOS) or quality of life among the survivors.

The sole purpose of surgical management in ICH is the following: (1) preventing a life-threatening brain herniation especially in young and salvageable patients and (2) improving hemiparesis in the putaminal bleed resulting from a compression of the internal capsule. The evacuation of hematoma means inadvertent collateral damage to the surrounding neural tracts while making surgical corridor to the hematoma. Therefore, more rational approach in assessing outcome would be through dichotomization of similar cohorts of patients with (1) putaminal bleed leading to compression of the internal capsule, confirmed by diffusion tensor imaging, and (2) those with significant hematoma with impending brain herniation and equivocal intracranial pressure, confirmed by assessing optic nerve sheath diameter, and patient randomization into surgical and nonsurgical management plans. Furthermore, among the patients opted for surgical management, further analysis with regards to variables, such as number of days on a ventilator and length of stay in intensive care unit, can also be undertaken among different approaches taken for hematoma evacuation, such as craniotomy, mini-craniotomy, or endoscopy. The use of only clinical scores such as modified Rankin Scale (mRS), Barthel Index (BI), or Glasgow Outcome score, while predicting clinical outcome, can sometimes give conflicting results. This may be exemplified by a small hematoma involving the internal capsule, which causes significant motor deficits (Morioka and Orito 2017).

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

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Mandibular Advancement Devices in Patients with Symptoms of Obstructive Sleep Apnea: A Review

M. Wojda, P. Jurkowski, A. Lewandowska,
E. Mierzwińska-Nastalska, and J. Kostrzewa–Janicka

Abstract

Obstructive sleep apnea (OSA) is a sleep disorder resulting from the repetitive narrowing and collapse of the upper respiratory tract. The results of previous epidemiological studies confirm a significant impact of OSA on the health situation around the world. Untreated OSA is associated with many adverse health effects, such as hypertension, coronary artery disease, stroke, atrial fibrillation, congestive heart failure, and daytime sleepiness. Excessive mortality of OSA patients, especially in men under 50 years of age, associated with advanced disease, obesity, cardiovascular complications, and a greater risk of road accidents, requires an urgent extension of the diagnostic–therapeutic database dealing with this problem. It is estimated that in the adult population, OSA occurs in 4% of men and in 2% of women. In recent years, intraoral devices have become an increasingly common method of OSA and snoring treatment. Nevertheless, the use of devices producing continuous positive airway pressure (CPAP) remains the most effective treatment method. However, intraoral devices have the advantage of not requiring a source of electricity and are less troublesome in everyday use. Intraoral devices are well tolerated by the majority of patients, and their therapeutic efficacy is confirmed. Since such devices become commoner, the purpose of this work was to present the procedures, indications, and recommendations involved with intraoral devices while taking into consideration a variety of dental conditions. The side effects of the use of intraoral devices and their influence on the entire stomatognathic system were also described.

Keywords

Continuous positive airway pressure · Intraoral device · Mandibular advancement appliance · Obstructive sleep apnea · Snoring · Stomatognathic system

1 Background

Obstructive sleep apnea (OSA) is characterized by repeated episodes of collapse (apnea) or narrowing of the upper respiratory tract (shallow breathing) at the level of the throat with preserved and in most cases increased, respiratory muscles work. The above episodes most often lead to a reduction in the oxygenation of arterial blood and usually result in waking up from sleep, although most awakenings remain unconscious. An

M. Wojda (✉), P. Jurkowski, A. Lewandowska,
E. Mierzwińska-Nastalska, and J. Kostrzewa–Janicka
Department of Prosthodontics, Warsaw Medical University, Warsaw, Poland
e-mail: monikawojda@op.pl

increase in the muscle tone of the upper respiratory tract and the sudden opening of the throat during awakening causes an increase in the vibration of soft tissues. This is manifested by very loud snoring during the restoration of proper breathing (Hudgel 1992). Untreated OSA is associated with many adverse health effects, such as hypertension, coronary artery disease, stroke, atrial fibrillation, congestive heart failure, and daytime sleepiness (Young et al. 2002).

The primary assessment in the everyday medical practice which allows for an unambiguous diagnosis of the disease is polysomnography (PSG). In turn, the sleepiness questionnaire developed in 1991 by Johns (1991) at the Epworth Hospital in Melbourne is simpler to implement, which is one of the criteria for diagnosing OSA. The Epworth Sleepiness Scale consists of eight questions concerning the possibility of falling asleep during specific life situations. The patient has a choice of four options: 0, no possibility of falling asleep, to 3, a high probability of falling asleep during a given situation. The sum of the points obtained can be from 0 to 24. Excessive daytime sleepiness is diagnosed when the sum of the points is $\geq$10.

Both conservative and surgical methods have been used to treat OSA. A change in lifestyle, weight loss, the discontinuation of drugs influencing breathing, as well as stopping alcohol and drug abuse are referred to as behavioral treatment and are also important for the effectiveness of treatment. A 10% reduction in body weight can result in a 50% decrease in the number of apneas and an increase in the arterial oxygen saturation. As a result, the sleeping pattern is also improved. In the case of anatomical abnormalities predisposing to the development of OSA or hypertrophic changes, surgical treatment is indicated (Hoffstein 2007; Padma et al. 2007; Sharples et al. 2016; Carra et al. 2012). The primary conservative treatment method for apnea is breathing with air delivered to the airways under positive pressure using a continuous airway pressure (CPAP). The beneficial effect of CPAP in patients with OSA is based on the pneumatic stiffening of the upper respiratory tract. The CPAP method is relatively safe; however, its long-term use is subject to certain complications. Patients treated with persistent positive airway pressure have local nasal injuries such as necrosis, irritation and mucosal edema, or nasal septum distortion. Approximately 40% of patients develop upper respiratory tract complaints, such as a runny nose, sneezing, and dryness of the mucous membrane. Often gas accumulates in the stomach as a result of swallowing air. Sometimes, the treatment may be ineffective and lead to the development of atelectasis. There may also be complications related to improperly fitting equipment (skin abrasions, sores, and irritations of the skin of the nose and conjunctiva), which results in the escape of air around the ill-fitting mask. The most serious complications that may arise during the use of this method include an intracranial embolism, bacterial meningitis, severe nosebleeds, subcutaneous edema, and arrhythmias. However, these are isolated and currently very rare cases (Standards of Practice Committee of American Sleep Disorders Association 1995).

Difficulties in accepting the CPAP treatment and the interest of other specialists in the subject of apnea allowed the introduction of new therapeutic solutions. These therapeutic methods aim at increasing the diameter of the upper respiratory tract by retracting the base of the tongue or protruding the mandible. This effect is provided by the mandibular advancement device (MAD), which functions by maintaining the mandible in a protruded position, displacing the tongue anteriorly via the genioglossus muscle, and changing the position of the hyoid bone, thus widening the upper respiratory tract (Sharples et al. 2016). It has been found that the use of MADs only for the stimulation of the genioglossus muscle without protruding the mandible does not affect the number of episodes of breathing obstructions during sleep (Fransson et al. 2002; Mehta et al. 2001).

The monoblock, which protrudes the mandible, was used for the first time by Robin (1934) in children with micrognathia. Currently, various types of devices are used to counteract

OSA. The devices may vary in construction, size, material, the way they adapt to teeth, the coverage of the teeth, and the possibility of vertical and lateral movements of the mandible. They can be standardized devices, prefabricated devices of the "boil and bite" system for self-adjustment by the patient or with the help of a dentist, devices with a smooth or step adjustment of the mandibular protrusion, or devices prepared individually for the patient. MAD appliances usually consist of two splints adapted to the shape of the dental arches that are placed on the teeth. Several randomized trials compared the effectiveness of different designs of devices that protrude the mandible (Ghazal et al. 2009; Lawton et al. 2005). Preliminary results of the studies show more favorable changes in both the clinical symptoms and the parameters assessed in polysomnography in the case of one-part devices (Bloch et al. 2000). However, 2-year observational studies show no differences in the long-term efficacy between single and two-part appliances (Ghazal et al. 2009). Two-part devices require an adaptation period consisting of gradually increasing the degree of mandibular protrusion up to an optimal therapeutic effect lasting up to even 8 weeks after a 4-week adaptation period. This long period before the full implementation of treatment is considered a disadvantage in cases where there is a need to quickly implement fully effective treatment. Kato et al. (2000) have presented a view that for every 2 mm of mandibular protrusion, there is an increase in the therapeutic effectiveness of the device by about 20%. Setting the mandible in a position that is 70% of the maximum protrusion is a compromise between the effectiveness of the device and its potential side effects. With regard to the vertical dimension of occlusion, it is believed that it should remain at a minimum level, because increasing the vertical dimension by opening the mandible leads to the tongue moving down and posteriorly, thereby reducing the airway patency (Pitsit et al. 2002; Bernhold and Bondemark 1998).

2 Indications, Contraindications, and Side Effects of Mandibular Advancement Devices (MAD)

The use of MAD treatment according to the Polish Society of Lung Diseases is indicated in patients with asocial snoring and a mild form of OSA, which does not improve after behavioral therapy. The American Academy of Sleep Medicine (AASM) and the American Academy of Dental Sleep Medicine (AADSM), akin to the British authors, indicate the use of dental appliances in patients with snoring without OSA, with mild OSA in combination with a reduction in the risk factors for sleep apnea, and with moderate-to-severe OSA in those who do not tolerate CPAP, do not express consent for their use, or do not qualify for surgical treatment (Standards of Practice Committee of American Sleep Disorders Association 1995). The guidelines recommend as a standard that sleep physicians consider the prescription of oral appliances, rather than no treatment, for adult patients. It is recommended that patients with severe OSA begin treatment with CPAP. Similarly, treatment with CPAP is more preferable than with MAD in patients requiring urgent treatment (e.g., drivers who fall asleep at the wheel) or patients with comorbidities, because CPAP is effective immediately, while MAD therapy requires an adaptive period until optimal therapeutic benefit is obtained (Kushida et al. 2006). It is suggested that a qualified dentist should make a custom, adjustable intraoral device and should follow up control visits in order to limit the side effects of therapy or occlusal changes. However, to improve sleep or confirm the effectiveness of MAD treatment, sleep medicine doctors should step in with control checkups (Ramar et al. 2015).

Before patients can be qualified for treatment with dental appliances and after they meet the criteria set out in the indications for MAD treatment, they should have a thorough extra- and intraoral examination. It is estimated that about one-third of OSA patients are excluded from MAD treatment solely on the basis of local dental

factors alone (Petit et al. 2002). The selection of patients in whom treatment with MAD could be effective is difficult due to a large number of factors determining treatment success using this method. There is a widespread belief that having a less severe form of OSA (Gotsopoulos et al. 2002; Mehta et al. 2001), a younger age, a lower body mass index (BMI), and a smaller neck circumference all improve the outcome of MAD treatment (Chung et al. 2010; Mehta et al. 2001). It is also believed that women respond better to this form of treatment (Marklund et al. 2004). In addition, morphological structure of the facial part of the skull and the physiology of the upper respiratory tract affect the therapeutic effect of MAD. On the basis of cephalometric studies, parameters related to the response to MAD treatment were determined. Better results of MAD treatment are achieved in patients with a longer maxilla, smaller overjet, shorter soft palate and facial height, reduced distance between the mandible and hyoid bone, and a smaller retropalatal airway space. It is believed that the success of treatment with MAD devices is affected by the distance from the posterior pharyngeal wall to the soft palate and from the angle formed by the ramus of the mandible with the line running through the sella turcica (Ng et al. 2012; Lee et al. 2010; Mehta et al. 2001; Liu and Lowe 2000). In addition, positive therapeutic effects of MAD are observed in patients whose upper airway collapses during sleep in the oropharyngeal region and in those with lower nasal resistance (Zeng et al. 2008; Ng et al. 2006).

In the qualifying clinical examination for MAD treatment, the number and quality of the remaining teeth, as well as the periodontal and temporomandibular joint health status should be assessed. The prerequisite for OSA treatment with MAD includes at least eight stable teeth in the maxilla and mandible and the ability to determine the centric occlusion with the position of the mandible in 50–75% of the maximum protrusion while leaving a space between the incisors of 3–5 mm that allows for free breathing through the mouth. The greater the mandibular protrusion, the greater is the effectiveness but also the poorer tolerance of the device. After determining the optimal protrusion of the mandible, it is recommended to evaluate the effectiveness of a device with a PSG test, because in some patients the dental prosthesis may increase the number of apneas (Johal and Bottegal 2001; Hans et al. 1997). MAD is contraindicated in cases of temporomandibular dysfunctions, including temporomandibular joint disorder, muscle complaints, lack of proper quantity and quality of teeth, and periodontal disease.

The side effects of MAD appliances have been divided into small and transient as well as moderate, severe, and chronic (Hoffstein 2007). Moderate, severe, and chronic adverse reactions prevent the continuation of treatment. The most common side effects are the following: excessive salivation, dry mouth, allergic reactions to the material used, and pain in the temporomandibular joints. Based on the cephalometric studies and model analysis, changes have been diagnosed after using MAD in the horizontal and vertical occlusal record, disturbances in Angle's classes (Angle 1907), and changes in the upper incisor angle to the base of the skull (1/NS) and in the angle between sella, nasion, and supramentale (SNB). In the studies assessing the use of Herbst devices, which protrude the mandible for 2 years, changes have been diagnosed in the position of incisors and a statistically insignificant reduction in vertical and horizontal occlusion, which was associated with the height of the splint but was unrelated to the extent of mandibular protrusion and to the length of time the devices were used (Battagel and Kotecha 2005; Fransson et al. 2003). In a study evaluating the effect of MAD on the stomatognathic system, Rinqqvist et al. (2003) used splints that did not cover the anterior part of the dental arches, and the mandibular protrusion in that case did not exceed 50%. The authors failed to observe vertical and horizontal occlusal changes or changes in the inclination angle of the upper and lower incisors. Marklund et al. (2004) observed fewer side effects in the case of devices made of elastic material. Bondemark and Lindman (2000) stated, however, that appliances made of hard material, thanks to the support of entire dental arches, are better for preventing occlusal changes. Martinez–Gomis

et al. (2010) showed that most dental changes occur within the first 2 years of use of mandibular protrusion devices.

3 Discussion

Based on the available medical evidence, OSA requires a multidisciplinary plan of treatment. Research confirms that devices that protrude the mandible can be an effective method of treatment in specific clinical cases, especially in early forms of the disease diagnosed on the basis of a clinical examination or in people with cardiovascular disease. Therapeutic efficacy of intraoral devices in OSA has been confirmed during the last decade by a significant number of randomized studies comparing MAD and CPAP modes of treatment (Kostrzewa-Janicka et al. 2016; Sharples et al. 2016; Quinnell et al. 2014; Gagnadoux et al. 2009; Hoffstein 2007; Fergusson et al. 1996). Studies provide the unambiguous evidence that the values of OSA indices decrease after the use of MAD or CPAP. Notably, AHI index decreases to a similar extent after treatment with MAD or CPAP, and these decreases are outstandingly significant in both treatment modes compared to placebo effects (Phillips et al. 2013; Hoekema et al. 2008; Barnes et al. 2004; Engelman et al. 2002; Tan et al. 2002). Likewise, excessive daytime sleepiness is clearly diminished using both treatment options (Aarab et al. 2011). Moreover, the improvements in the patient's condition are long-lasting as they are sustained for up to a 2-year-long follow-up using either treatment option (Doff et al. 2013). These results encourage the use of MAD as an efficacious alternative to CPAP therapy in patients with mild-to-moderate OSA. However, in severe OSA, the treatment of choice remains to be CPAP.

Compliance with indications and contraindications to MAD treatment is indispensable for achieving the intended therapeutic effects, while protecting the patient from adverse effects. A simple construction of MAD devices, their availability, and the comfort of use encourage a widespread application among people with the symptoms of snoring or with milder forms of OSA, who do not tolerate CPAP treatment. After the implementation of therapy with an intraoral device, control visits at the dental office in order to assess the masticatory motor system are necessary. The effectiveness of OSA treatment should be objectively evaluated by repeat PSG examinations.

Conflict of Interest The authors declare no conflict of interest in relation to this article.

Ethical Consideration This review article does not contain any studies with human participants or animals performed by any of the authors.

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Online Health Technologies and Mobile Devices: Attitudes, Needs, and Future

Joanna Waligóra and Maria Magdalena Bujnowska–Fedak

Abstract

Advances in mobile technology constitute a promising and evolving trend that enables better access to health care especially for the elderly, disabled, and chronically ill. It overcomes geographical, temporal, and organizational barriers at low and affordable costs. The aim of the study was to evaluate the needs and expectations of Polish citizens and their attitudes toward mobile health (mHealth) services using mobile phones and communication devices in medical care and also to evaluate the sociodemographic factors affecting such behavioral processes. A total of 1000 adults were selected from the Polish population by random sampling. The assessment was made with the use of computer-assisted telephone interview (CATI). Approximately 78% of the study participants were proficient mobile phone users with a predominance of young people. Forty-seven percent of them expressed the desire to obtain information about their health via their mobile phone if they had the opportunity to do so. Important factors associated with the aforementioned statement included younger age, being still in education, or unemployed. Among the mHealth supporters, the vast majority of people (84%) would like to receive SMS (short message service) reminders for appointments and prescribed medicines. Other favorable mHealth activities were e-registration (77.9%), viewing test results online (80.6%), or receiving basic medical recommendations (75.7%). Only 30% of the respondents had a positive attitude toward teleconsultation, while 17.8% of them were willing to pay for this option. Further research on emerging new and beneficial mHealth solutions needs to be conducted.

Keywords

E-health · Medical informatics · Mobile health service · Mobile phone · Sociodemographic factors

1 Introduction

Digital transformation has revolutionized almost every aspect of our lives and has the potential to increase health-care quality across the globe. Mobile health technology, commonly known as mHealth, is a relatively recent development in the digital world. It includes using devices such as cell phones, iPads, tablets, laptops, and similar devices in order to obtain access to health information networks. Using a smartphone or tablet for health management has now become as important as the traditional use of websites with a computer. The

J. Waligóra and M. M. Bujnowska–Fedak (✉)
Department of Family Medicine, Wroclaw Medical University, Wroclaw, Poland
e-mail: mbujnowska@poczta.onet.pl

results of a worldwide survey including 7905 consumers published in 2018 by Accenture showed that for health management, 50% of respondents used websites and 48% used smartphones or tablets (Accenture Consumer Survey 2018).

Advances in mobile technology constitute a promising and evolving trend. They enable better access to health care, especially for the elderly, disabled, and chronically ill. They overcome geographical, temporal, and organizational barriers at low and affordable costs. Given their inexpensiveness, these devices are becoming increasingly available. The number of mobile phone users worldwide is growing rapidly. In 2010, 296 million smartphones were sold worldwide (Gartner 2011). In 2013, more than one billion smartphones were delivered to global markets. In the same year, there was a higher sale of smartphones than traditional cell phones (Business Wire 2014). According to a US national study, 95% of Americans own a mobile phone of some kind. In 2011, just 35% of Americans had a smartphone, while in 2018 this percentage was 77%. The ownership of a mobile phone is common across a wide range of demographic groups. By contrast, smartphone ownership depends on a greater variation of factors based on age, household income, and educational attainment (Pew Research Center 2018).

Smartphones allow real-time and on-demand communication, they store and exchange large amounts of personal information, and – through their multimedia-rich touch displays – they facilitate the lives of their owners. Thanks to the possibility of data storage in smartphones, mHealth applications enable the collection of substantial amounts of medical data, data on physiology, lifestyle, and daily activities. Typical examples of such applications include medical education materials and motivational tools such as reminding about taking medications or offering advice on exercises and diets (Boulos et al. 2014). A smartphone with Internet access and other built-in features like text messaging, e-mailing, web browsing, camera, GPS, audio, and video is crucial for the operation of mHealth services. These built-in devices provide additional possibilities of the implementation of mHealth applications. As a result, mHealth solutions may improve the efficiency of health protection both in the area of therapy and in the management of the health system. Telemonitoring in chronic diseases and issuing e-prescriptions during remote consultations are becoming more and more popular. Thanks to such solutions, users have easier access to information on their health at any place and time (Gensini et al. 2017).

Mobile devices and applications (apps) provide numerous benefits to the health-care system. These increasingly sophisticated tools could be a solution in favor of supporting and promoting patient care. In order to maximize the value and proper incorporation of these tools into health-care systems, their impact on their users must be well known. Therefore, the aim of this study was to evaluate the attitudes, needs, and expectations of Polish citizens with regard to selected mHealth services and to determine the sociodemographic factors affecting such behavioral processes.

2 Methods

2.1 Participants

A random sample of 1000 Polish adults was chosen nationwide to be included in the survey carried out between December 2017 and January 2018. The attitude toward mHealth services using mobile phones and communication devices in medical care was assessed by means of computer-assisted telephone interview (CATI). To make sure that the study group was representative, the province and town/city size were considered for the determination of the geographical distribution of respondents, who were further selected based on sociodemographic characteristics. On average, 5.2% of subjects agreed to participate in the study. Those who refused to do so, who did not answer the phone, who were too sick to participate, or whose phone number was incorrect fell into the nonresponders' group. The majority of the nonresponders did not give any reason for their refusal. As a result, they were replaced by another household from the same region or town/city until 1000 participants completed the questionnaire.

2.2 Questionnaire

A questionnaire was designed to identify and determine diverse aspects related to the use of the Internet for health-related purposes and general opinion on telemedicine applications. In this study, we only focused on questions concerning consumer attitudes to, and views on, mobile health apps and associations between them and sociodemographic characteristics, including health status. First, the respondents were asked whether they had a mobile phone and were proficient in the use of a smartphone. Among those who gave a positive answer to this question, the respondents were further divided into two groups: supporters and non-supporters of mHealth use. In the supporters' group, the opinion on the following mHealth services was measured: SMS (short message service) reminders about medical appointments or taking medications, teleconsultation, telemonitoring, medical test results reporting on cell phones, online registration, obtaining simple medical recommendations, and others. In addition, the participants were asked about their general opinion on the remote consultation and willingness to pay for it. The questionnaire also included items related to sociodemographic characteristics and health conditions such as respondent's age, gender, education, and place of residence.

2.3 Data Analysis

In order to notice significant correlations between sociodemographic factors of the participants and their attitude toward mobile health apps, both descriptive and statistical analyses were incorporated. The Shapiro–Wilk test was used to check quantitative variables for normality of distribution which none of them displayed. Therefore, only nonparametric tests were used to carry out the analysis. The Wilcoxon multiple comparison test was conducted to compare the distribution of quantitative variables between the groups. For qualitative variables, the Chi-squared and Fisher's independence tests were used to determine statistically significant dependencies. For all tests, the significance level was assumed to be 0.05. The R statistical package (version 3.5.1) was used for calculations.

3 Results

3.1 Characteristics of Respondents

The study included 558 women and 442 men randomly selected from among the Polish population. The median age was 53 (min–max, 18–88) years. There were 957 persons who declared that they used a mobile phone, mostly smartphones, but only 778 (77.8%) of them were proficient in its use. Six hundred and thirteen (61.3%) of respondents lived in cities/towns and 387 (38.7%) in rural areas. The majority of 856 (85.6%) respondents lived with someone else, while another 144 (14.4%) lived alone. As for employment status, 594 (59.4%) of respondents were professionally active, 317 (31.7%) were retirees or chronically ill/disabled, and 44 (4.4%) were still in education. Three hundred thirty-nine (33.9%) of respondents completed primary, 373 (37.3%) secondary, and 288 (28.8%) higher education. More than half of respondents were in good/very good health (n = 570; 57.5%), less in fair health ($n = 347$; 35%), and only 74 (7.5%) in poor/very poor health.

3.2 Factors Affecting Mobile Phone Use

Persons who were dexterous in using mobile phone devices were usually younger ($p < 0.0001$), better educated ($p < 0.0001$), and in better health ($p < 0.0001$). Those who were professionally active or still in education fully embraced this technology more frequently compared to those who were retired or chronically ill ($p < 0.0001$). What is more, a significantly greater percentage of mobile phone users lived in cities/towns ($p = 0.002$) and with their families or with someone else but not alone ($p = 0.002$).

According to the questionnaire, women and men equally benefit from mobile devices. For more information, see Table 1.

3.3 Support for Mobile Health (mHealth) Activities

Forty-seven percent of mobile phone users ($n = 366$) supported mHealth and 53% ($n = 412$) did not. Younger age increased the probability of having a positive attitude toward mHealth services ($p < 0.00001$). Further, people who were still in education or unemployed were more willing to support it ($p < 0.0001$) (Table 2). The most common mHealth activity was SMS reminders. In the group of SMS-reminder supporters, those who more frequently lived with their families ($p < 0.05$) and those who were still students, professionally active, or unemployed prevailed significantly ($p < 0.05$). People who opted for online registration were younger ($p < 0.05$), more often students or with completed higher education ($p = 0.01$), professionally active or unemployed ($p = 0.01$), and in better health status ($p < 0.05$). The same sociodemographic factors influenced the attitude toward mobile phone teleconsultation. Younger age had a positive effect on receiving test results on mobile phones ($p = 0.01$). There was no statistically significant correlation between telemonitoring, getting simple medical recommendations, and sociodemographic factors. For more details, see Table 3.

Table 1 Mobile phone users

Characteristics	Proficient mobile phone users *n* (%)	Non-proficient mobile phone users *n* (%)
All	778 (77.8)	222 (22.2)
Men	353 (79.9)	89 (20.1)
Women	425 (76.2)	133 (23.8)
Age groups (years)		
18–35	278 (92.1)	24 (7.9)**
36–59	360 (83.9)	69 (16.1)
60+	140 (52.0)	129 (48.0)
Education		
Basic/vocational	208 (61.4)	131 (38.6)**
Secondary	310 (83.1)	63 (16.9)
Higher/some higher	260 (90.3)	28 (9.7)
Employment status		
Education process	43 (97.7)	1 (2.3)**
Paid work/voluntary/others	536 (90.2)	58 (9.8)
Retired/permanently disabled	166 (52.4)	151 (47.6)
Unemployed	33 (73.3)	12 (26.7)
Residency type		
Alone	96 (67.1)	47 (32.9)*
Family/others	681 (79.6)	175 (20.4)
Residency place		
Urban	497 (81.1)	116 (18.9)*
Rural	281 (72.6)	106 (27.4)
Health status		
Good/very good	498 (87.4)	72 (12.6)**
Fair	232 (66.9)	115 (33.1)
Poor/very poor	41 (55.4)	33 (44.6)

*$p = 0.002$; **$p < 0.0001$ for intergroup comparisons

Table 2 mHealth supporters vs. non-supporters

Characteristics	mHealth supporters *n* (%)	mHealth non-supporters *n* (%)
All	366 (47.1)	412 (52.9)
Men	172 (48.7)	181 (51.3)
Women	194 (45.6)	231 (54.4)
Age groups (years)		
18–35	151 (54.3)	127 (45.7)*
36–59	170 (47.2)	190 (52.8)
60+	45 (32.1)	95 (67.9)
Education		
Basic/vocational	93 (44.7)	115 (55.3)
Secondary	142 (45.8)	168 (54.2)
Higher/some higher	131 (50.4)	129 (49.6)
Employment status		
Education process	26 (60.5)	17 (39.5)*
Paid work/voluntary/others	266 (49.6)	270 (50.4)
Retired/permanently disabled	55 (33.1)	111 (66.9)
Unemployed	19 (57.6)	14 (42.4)
Residency type		
Alone	53 (55.2)	43 (44.8)
Family/others	313 (46.0)	368 (54.0)
Residency place		
Urban	241 (48.5)	256 (51.5)
Rural	125 (44.5)	156 (55.5)
Health status		
Good/very good	247 (49.6)	251 (50.4)
Fair	97 (41.8)	135 (58.2)
Poor/very poor	21 (51.2)	20 (48.8)

*$p < 0.0001$ for intergroup comparisons

3.4 Opinion About Teleconsultation

Merely a third (29.7%) of respondents adopted a positive attitude toward mobile phone remote consultations. There were 61.3% of the proficient mobile phone users and 84.5% of the mHealth supporters who expressed their willingness for mobile phone teleconsultations (Fig. 1). Only 17.8% of respondents were ready to pay €10 for it. Compared to the other groups, the attitude of mHealth supporters toward paying for mobile phone teleconsultations was the most positive. The highest rate of a favorable attitude toward mobile phone teleconsultations was most frequently noticed among young people ($p < 0.0001$), with a high level of education ($p < 0.0001$), who lived in cities/towns ($p < 0.03$), and who were still in the process of education ($p = 0.004$) and in good health ($p < 0.03$). The willingness to pay for teleconsultation was correlated with younger age ($p < 0.0001$), a high level of education ($p < 0.0001$), being unemployed or professionally active ($p < 0.0001$), and being in good health ($p < 0.0001$) (Table 4).

4 Discussion

Over the years, mobile technology has witnessed many improvements that made mobile phones available for anyone everywhere. Due to the advanced computing and communication capability, which includes Internet access and global positioning systems, there is a great potential for

Table 3 Factors affecting mHealth services

	SMS reminders		Medical test result reporting		Online registration		Getting simple medical advice		Telemonitoring		Teleconsultation		Others	
	n (%)		*n* (%)		*n* (%)o		*n* (%)		*n* (%)		*n* (%)		*n* (%)	
Characteristics	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
All	309 (84.4)	57 (15.6)	295 (80.6)	71 (19.4)	285 (77.9)	81 (22.1)	277 (75.7)	89 (24.3)	204 (55.7)	162 (44.3)	160 (43.7)	206 (56.3)	22 (6.0)	344 (94.0)
Men	140 (81.4)	32 (18.6)	134 (77.9)	38 (22.1)	129 (75.0)	43 (25.0)	128 (74.4)	44 (25.6)	101 (58.7)	71 (41.3)	76 (44.2)	96 (55.8)	11 (6.4)	161 (93.6)
Women	169 (87.1)	25 (12.9)	161 (83.0)	33 (17.0)	156 (80.4)	38 (19.6)	149 (76.8)	45 (23.2)	103 (53.1)	91 (46.9)	84 (43.3)	110 (56.7)	11 (5.7)	183 (94.3)
Age group (years)														
18–35	131 (86.8)	20 (13.2)	122 (80.8)	29 (19.2)**	121 (80.1)	30 (19.9)*	111 (73.5)	40 (26.5)	77 (51.0)	74 (49.0)	66 (43.7)	85 (56.3)	3 (2.0)	148 (98.0)**
36–59	144 (84.7)	26 (15.3)	144 (84.7)	26 (15.3)	136 m (80.0)	34 (20.0)	136 (80.0)	34 (20.0)	100 (58.9)	70 (41.2)	78 (45.9)	92 (54.1)	15 (8.8)	155 (91.2)
60+	34 (75.6)	11 (24.4)	29 (64.4)	16 (35.6)	28 (62.2)	17 (37.8)	30 (66.7)	15 (33.3)	27 (60.0)	18 (40.0)	16 (35.6)	29 (64.4)	4 (8.9)	41 (91.1)
Education														
Basic/vocational	79 (84.9)	14 (15.1)	71 (76.3)	22 (23.7)*	63 (67.7)	30 (32.3)**	69 (74.2)	24 (25.8)*	55 (59.1)	38 (40.9)	32 (34.4)	61 (65.6)***	7 (7.5)	86 (92.5)
Secondary	119 (83.8)	23 (16.2)	110 (77.5)	32 (22.5)	110 (77.5)	32 (22.5)	100 (70.4)	42 (29.6)	78 (54.9)	64 (45.1)	54 (38.0)	88 (62.0)	8 (5.6)	134 (94.4)
High	111 (84.7)	20 (15.3)	114 (87.0)	17 (13.0)	112 (85.5)	19 (14.5)	108 (82.4)	23 (17.6)	71 (54.2)	60 (45.8)	74 (56.5)	57 (43.5)	7 (5.3)	124 (94.7)

Employment status														
Education process	23 (88.5)	3 (11.5)*	19 (73.1)	7 (26.9)*	19 (73.1)	7 (26.9)**	18 (69.2)	8 (30.8)	15 (57.7)	11 (42.3)	5 (19.2)	21 (80.8)*	1 (3.8)	25 (96.2)*
Paid work/ voluntary work/others	231 (86.8)	35 (13.2)	223 (83.8)	43 (16.2)	218 (82.0)	48 (18.0)	210 (78.9)	56 (21.1)	145 (54.5)	121 (45.5)	127 (47.7)	139 (52.3)	12 (4.5)	254 (95.5)
Unemployed	16 (84.2)	3 (15.8)	15 (78.9)	17 (30.9)	14 (73.7)	5 (26.3)	13 (68.4)	6 (31.6)	13 (68.4)	24 (31.6)	7 (36.9)	12 (63.2)	1 (5.3)	18 (94.7)
Retirement/ Permanently disabled	39 (70.9)	16 (29.1)	38 (69.1)	4 (21.1)	34 (61.8)	21 (38.2)	36 (65.5)	19 (34.5)	31 (56.4)	6 (43.6)	21 (38.2)	34 (61.8)	8 (14.5)	47 (85.5)
Residency type														
Alone	39 (73.6)	14 (26.4)*	39 (73.6)	14 (26.4)	41 (77.4)	12 (22.6)	38 (71.7)	15 (28.3)	30 (56.6)	23 (43.4)	25 (47.2)	28 (52.8)	3 (5.7)	50 (94.3)
Family	270 (86.3)	43 (13.7)	256 (81.8)	57 (18.2)	244 (78.0)	69 (22.0)	239 (76.4)	74 (23.6)	174 (55.6)	139 (44.4)	135 (43.1)	178 (56.9)	19 (6.1)	294 (93.9)
Residence place														
Urban	202 (83.8)	39 (16.2)	193 (80.1)	48 (19.9)	189 (78.4)	52 (21.6)	177 (73.4)	64 (26.6)	131 (54.4)	110 (45.6)	106 (44.0)	135 (56.0)	14 (5.8)	227 (94.2)
Rural	107 (85.6)	18 (14.4)	102 (81.6)	23 (18.4)	96 (76.8)	29 (23.2)	100 (80.0)	25 (20.0)	73 (58.4)	52 (41.6)	54 (43.2)	71 (56.8)	8 (6.4)	117 (93.6)
Health status														
Good/very good	215 (87.0)	32 (13.0)	202 (81.8)	45 (18.2)	201 (81.4)	46 (18.6)*	190 (76.9)	57 (23.1)	133 (53.8)	114 (46.2)	113 (45.7)	134 (54.3)**	12 (4.9)	235 (95.1)*
Fair	75 (77.3)	22 (22.7)	75 (77.3)	22 (22.7)	67 (69.1)	30 (30.9)	70 (72.2)	27 (27.8)	55 (56.7)	42 (43.3)	33 (34.0)	64 (66.0)	6 (6.2)	91 (93.8)
Bad/very bad	18 (85.7)	3 (14.3)	17 (81.0)	4 (19.0)	17 (81.0)	4 (19.0)	17 (81.0)	4 (19.0)	16 (76.2)	5 (23.8)	14 (66.7)	7 (33.3)	4 (19.0)	17 (81.0)

*$p < 0.05$; **$p = 0.01$; ***$p < 0.001$ for intergroup comparisons

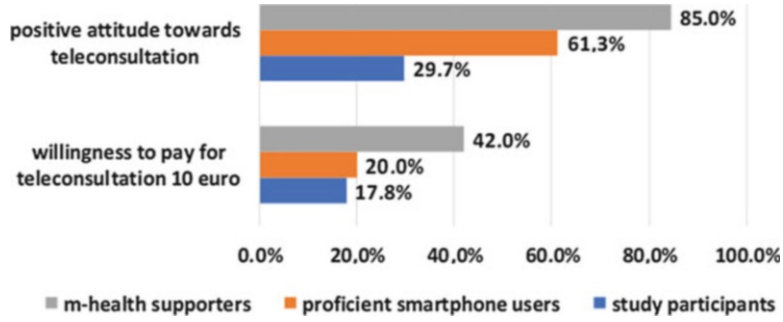

Fig. 1 General attitude toward mobile phone teleconsultation and willingness to pay for the service

Table 4 Factors affecting attitudes toward mobile phone teleconsultation

	Positive attitude toward mobile phone teleconsultation	Negative attitude toward mobile phone teleconsultation	Willingness to pay for teleconsultation	Unwillingness to pay for teleconsultation
Characteristics	*n* (%)	*n* (%)	*n* (%)	*n* (%)
All	297 (29.7)	703 (70.3)	178 (17.8)	822 (82.2)
Men	142 (32.1)	300 (67.9)	87 (19.7)	355 (80.3)
Women	155 (27.8)	403 (72.2)	91 (16.3)	467 (83.7)
Age group (years)				
18–35	112 (37.1)	190 (62.9)****	71 (23.5)	231 (76.5)****
36–59	123 (28.7)	306 (71.3)	78 (18.2)	351 (81.8)
60+	62 (23.0)	207 (77.0)	29 (10.8)	240 (89.2)
Education				
Basic/ vocational	81 (23.9)	258 (76.1)****	42 (12.4)	297 (87.6)***
Secondary	106 (28.4)	267 (71.6)	66 (17.7)	307 (82.3)
High	110 (38.2)	178 (61.8)	70 (24.3)	218 (75.7)
Employment status				
Education process	13 (29.5)	31 (70.5)**	4 (9.1)	40 (90.9)****
Paid work/ voluntary work/ others	189 (31.8)	405 (68.2)	130 (21.9)	464 (78.1)
Unemployed	21 (46.7)	24 (53.3)	11 (24.4)	34 (75.6)
Retirement/ permanently disabled	74 (23.3)	243 (76.7)	33 (10.4)	284 (89.6)
Residency type				
Alone	45 (31.5)	98 (68.5)	25 (17.5)	118 (82.5)
Family	252 (29.4)	604 (70.6)	153 (17.9)	703 (82.1)
Residence place				
Urban	198 (32.3)	415 (67.7)*	118 (19.2)	495 (80.8)
Rural	99 (25.6)	288 (74.4)	60 (15.5)	327 (84.5)
Health status				
Good/very good	190 (33.3)	380 (66.7)*	125 (21.9)	445 (78.1)****
Fair	87 (25.1)	260 (74.9)	49 (14.1)	298 (85.9)
Bad/very bad	20 (27.0)	54 (73.0)	4 (5.4)	70 (94.6)

*$p < 0.03$; **$p < 0.01$; ***$p = 0.004$; ****$p < 0001$ for intergroup comparisons

application development. These technological innovations are implemented to improve access to, quality, and experience concerning just about every social, entertainment, and educational area of our lives. It is inevitable that we would also turn to digital solutions in the face of medical problems.

mHealth devices and telehealth platforms are increasingly used to complement medical care for patients who are proficient in the use of the Internet and other electronic communication tools. To use them accurately and appropriately, it is necessary for patients to be competent in digital and mobile technology. In the present study, over three fourths of the people were proficient mobile phone users. Nearly half of them were interested in mHealth. For the comparison of our current results to some prior studies, a 2012 Pew Research Center survey found that only 19% of mobile phone users had at least one health app (Fox and Duggan 2012). In turn, a German population-based survey conducted in 2017 revealed that among mobile phone users, around 20% used health apps (Ernsting et al. 2017). This study indicates a growing interest in various mHealth services. It is young people who were professionally active, with higher education, or still in education who were especially willing to use health apps for cell phones. Young age, a high level of education, and higher earnings were the key sociodemographic factors that were positively correlated with mHealth support in other studies (Carroll et al. 2017; Bhuyan et al. 2016; Krebs and Duncan 2015). However, there are still obstacles to a widespread use of mHealth technology. Technical systems may be poorly adapted to some groups of recipients. As this study showed, people who are old, retired, or chronically ill and lived alone and in rural areas were the least willing to use mobile devices. Nevertheless, it seems that it is these very groups that should reap the greatest benefits of telemedicine such us easier access to specialists, remote monitoring, and providing quick actions in case of emergency (van Houwelingen et al. 2018; Kaambwa et al. 2017; Bujnowska–Fedak 2015). A positive correlation was observed between living with the family and the willingness to use cell phones. This may indicate that when it comes to technology efficiency, it is beneficial for older generations to live with younger people. People are glad to ask their relatives for help in using the latest technologies (Zickuhr 2013). However, training in the field of using mobile technologies is required to allow them to take full advantage of benefits of mHealth.

The development and adoption of new methods of communication provide new opportunities for delivering health services. Due to the ubiquity of text messaging, rapid and automated delivery, and its relatively low cost, SMS has become recommended for use by leading organizations in various health-care fields (Schwebel and Larimer 2018). SMS reminders were primarily focused on outcomes such as appointment attendance and medication adherence (Berrouiguet et al. 2016; Kannisto et al. 2014). Currently, they may also serve as coaching tips or simple medical recommendations to support the desirable change of behavior (Prochaska et al. 1994; Fjeldsoe et al. 2009). This study confirmed the highest willigness of mobile phone users toward this service (88% of the mHealth supporters), which is very encouraging. A recent meta-analysis of randomized controlled trials demonstrates that medication adherence among patients with chronic conditions increases twofold with mobile phone text messaging (Thirumurthy and Lester 2012). A particular advantage of SMS services lies in that that they usually do not contain sensitive personal data. As it is well known, the security and confidentiality of data are a big concern for users (Suslo et al. 2018). Online registration and medical test results reporting also received a highly positive feedback in the current study (77.9% and 80.6% of the mHealth supporters, respectively). Medical appointment scheduling may improve the operation of the health-care system by improving access to it, decreasing waiting time, and decreasing staff labor (Zhao et al. 2017). Consequently, medical test results reporting makes it possible to provide a seamless exchange of information between the doctor and the patient. Using an app is faster and easier than downloading and printing lab results (Dullabh et al. 2014).

Teleconsultations and remote monitoring tools met with cautious response (43.7% and 55.7% of the mHealth supporters, respectively). People are still unwilling to change direct face-to-face communication with the doctor. Young age significantly affected the likelihood of using teleconsultation. According to a study by Zocdoc (2015), people who are raised in the digital era are less likely to visit the doctor and more naturally are inclined to make contact online instead. As reported by Krebs and Duncan (2015), a financial aspect is also of considerable importance in terms of the use of health apps. Approximately half of the app users stop using certain health apps due to hidden costs, which is consistent with the results of the current study. We noticed that a mere 17.8% of the entire cohort was willing to pay for teleconsultation. Yet the rate of people who were prepared to pay for the service significantly increased (42%) in the group of the proficient mHealth users. This is an important finding as it offers the opportunity to increase the profitability of mHealth due to cost-effective telemedical solutions.

This study has several limitations. The response rate was low (5.2% on average), albeit that is a rather usual finding in this type of study. Nonetheless, a nonresponse bias could have occurred and affected the estimates. In addition, the December–January time of the audit, which includes a holiday season and a winter break, might have influenced the low response rate. Moreover, due to the nature and high speed of the telephone conversation in CATI, the respondents were deprived of the possibility of thinking about and giving the most appropriate answer. The question about the financial status of respondents was also missing. On the other hand, it seems inappropriate to ask about earnings during a telephone interview, which might have given unreliable answers. Lastly, it is worth mentioning that this study focused on the attitudes of users rather than on patients' outcomes. Therefore, the exact rate of the use of particular mHealth services is unknown. Further studies are necessary to assess the actual use of health apps rather than the attitudes and perceptions of their users.

5 Conclusions

Mobile technology has the potential to make the health-care system more efficient, less expensive, and more accessible. mHealth devices and telehealth platforms support disease management for patients who are proficient in the use of these tools. As with any new technology, the adaptation to mHealth services is a work in progress. Those who are young, with higher education, and who are professionally active are more drawn to digital technology than the rest of the population. Those who are older, retired, and chronically ill or disabled struggle to adapt to digital technology. Initial fears and the lack of willingness to use it at the beginning do not mean that it is ineffective. Even if the implementation of mHealth services is difficult to do well in certain environments, it is crucial to provide advice and to encourage potential users to benefit from it. The article demonstrates the need for further research to be performed to show the effects of numerous innovative uses of mobile technology on health outcomes.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Respondents were provided with comprehensive information on the objectives and scope of survey and gave their informed consent. The survey was approved by the Bioethics Committee of Wroclaw Medical University in Poland.

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Radiological Response and Neutrophil-to-Lymphocyte Ratio as Predictive Factors for Progression-Free and Overall Survival in Metastatic Renal Cell Carcinoma Patients Treated with Sunitinib

Jakub Kucharz, Paulina Dumnicka, Agnieszka Giza, Urszula Demkow, Beata Kusnierz–Cabala, Tomasz Demkow, and Pawel Wiechno

Abstract

Renal cell carcinoma (RCC) represents 2–3% of all malignancies. Most RCC-related deaths are caused by metastases of the disease. Studies suggest that inflammation-related parameters are of prognostic significance in metastatic renal cell carcinoma (mRCC) patients. Neutrophilia and thrombocytosis are markers of systemic inflammation that accompanies cancer, while lymphopenia is related to dysfunctions of the immune system. Neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) thus seem particularly interesting from a clinical perspective. The goal of this study was to determine if the response to therapy, consisting of reductions in radiologically assessed tumor burden and in inflammation-related parameters after 12 weeks of treatment with sunitinib, has a predictive value for outcome. One hundred thirty-one mRCC patients treated with the first-line sunitinib were evaluated. Inflammation-related parameters and radiologic response were correlated with treatment outcomes, progression-free, and overall survival. We found that the longest median progression-free survival of 37 months (Q1; Q3–15; not reached) and overall survival of 40 months (Q1; Q3–26; not reached) were achieved by patients who had either partial or complete response according to RECIST 1.1 and NLR lower than 1.64. In conclusion, the study confirmed that both objective response and lower grade of inflammation during

J. Kucharz (✉), T. Demkow, and P. Wiechno
Department of Urooncology, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland
e-mail: jakub.kucharz@coi.pl

P. Dumnicka
Department of Medical Diagnostics, Jagiellonian University Medical College, Cracow, Poland

A. Giza
Department of Hematology, Jagiellonian University Medical College, Cracow, Poland

U. Demkow
Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Warsaw Medical University, Warsaw, Poland

B. Kusnierz–Cabala
Department of Diagnostics, Chair of Clinical Biochemistry, Jagiellonian University Medical College, Cracow, Poland

treatment are predictive of better outcomes in mRCC patients treated with sunitinib.

Keywords
Cancer · Metastases · Neutrophil-to-lymphocyte ratio · Radiological response · Renal cell carcinoma · Sunitinib · Survival

1 Introduction

Renal cell carcinoma (RCC) represents 2–3% of all malignancies. Over 175,000 patients die of RCC every year (Bray et al. 2008). Most RCC-related deaths are caused by metastases. Metastatic disease is found in approximately 30% of patients at initial diagnosis and in further 20% of those who completed curative treatment (Athar and Gentile 2008; Gupta et al. 2008). The risk of developing metastasis after radical treatment can be assessed using nomograms and scoring systems that incorporate pathologic stage, tumor size, lymph node status, and the presence of necrosis (Leibovich et al. 2003). Scoring systems have been developed for metastatic disease to facilitate prognosis. The most frequently used scales are those elaborated by the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) and by the Memorial Sloan Kettering Cancer Center (MSKCC) (Motzer et al. 1999; Motzer et al. 2013; Heng et al. 2009).

It is worth noting that the IMDC scale includes factors related to inflammation, which manifests in the elevated platelet and neutrophil counts. Studies also suggest that the prognosis in metastatic RCC (mRCC) is connected with pro-inflammatory markers such as C-reactive protein (CRP) (Tatokoro et al. 2008) or interleukin-6 (Ljungberg et al. 1997). Neutrophilia, monocytosis, and thrombocytosis are markers of systemic inflammation that accompanies cancer, while lymphopenia is related to dysfunction of the immune system (Grivennikov et al. 2010). Neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and lymphocyte-to-monocyte ratio (LMR) seem particularly interesting from the clinical perspective. Some studies suggest that elevated NLR (Zhang et al. 2016, 2018; Xiao et al. 2014; Kishi et al. 2009), PLR (Zhou et al. 2014), and LMR (Gu et al. 2016) are associated with poor prognosis in malignancies, including mRCC.

Significant progress has been made in systemic RCC treatment. A proper qualification for therapy plays a key role. For patients with intermediate and poor prognosis, as determined by the IMDC, combined nivolumab and ipilimumab immunotherapy is poised to become standard of care as it leads to the longest overall survival. Sunitinib therapy, on the other side, has led to better outcomes than immunotherapy in patients with the IMDC-assessed favorable prognosis. Recently, sunitinib has become a standard for comparison of other first-line treatments in most clinical trials (Motzer et al. 2018).

Cabozantinib, another medication registered as a first-line treatment agent, has been tested in a second-phase study in patients with intermediate or poor prognosis. This medication prolongs progression-free survival but not overall survival (Choueiri et al. 2018). There are reasons to question the use of tyrosine kinase inhibitors (TKI), exerting influence on the vascular endothelial growth factor (VEGF), and anexelekto (AXL) and MET downstream signaling pathways, in the first-line treatment. Such a broad mechanism of action may limit the choice of subsequent treatments. Sunitinib is a receptor protein-tyrosine kinase inhibitor and its use is related to better outcome in patients with the IMDC-assessed favorable prognosis. This medication continues to play a significant role in first-line mRCC treatment. However, sunitinib treatment also is associated with adverse events, which affect patients' quality of life and cause comorbidities. The early assessment of prognosis is thus essential to obtain the optimum therapeutic effects.

Achievement of complete or partial radiological response to the treatment has been shown to be associated with better prognosis in mRCC (Molina et al. 2014; Seidel et al. 2013), accompanied by amelioration of immune and inflammatory-related dysregulation (Lalani et al. 2018; Templeton et al. 2016; Wu et al. 2016; Lee

et al. 2013). Therefore, the present study addresses the issue of whether the response to therapy consisting of reductions in radiologically assessed tumor burden and in inflammation-related parameters, after 12-week-long treatment could be predictive of better outcome in mRCC patients who were first-line treated with sunitinib.

2 Methods

2.1 Patient Selection and Therapeutic Procedure

This retrospective study included 131 consecutive adult patients with mRCC, treated with sunitinib as the first-line treatment between 2011 and 2017. Inclusion criteria were the following: prior nephrectomy (total or nephron-sparing surgery) for RCC, predominantly clear cell histology, metastatic disease or non-resectable local recurrence, and a favorable or intermediate-risk score on the Memorial Sloan Kettering Cancer Center (MSKCC) scale (Motzer et al. 1999). All patients received sunitinib in a standard dose regimen, i.e., 50 mg/day, 4 weeks on/2 weeks off. Patients were treated until they either reached a point of disease progression according to the response evaluation criteria in solid tumors (RECIST) 1.1 criteria (Eisenhauer et al. 2009) or they could no longer tolerate toxicity. Dose reductions were based on the Common Criteria for Adverse Events (CTCAE 2010).

Data were collected from the patients' medical records and included the following features:

- Demographics: age and body mass index (BMI) before the first cycle of sunitinib treatment (at time 0; t_0), gender, disease stage at diagnosis, Fuhrman grade, time from diagnosis to systemic treatment, performance score at onset of treatment, sites and number of metastases, and the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) (Heng et al. 2009) and MSKCC prognosis scores at onset of treatment.
- Results of radiologic assessment performed at week 12 (t_2) and treatment response classified according to RECIST 1.1 criteria.
- Results of a complete blood count (CBC), with leukocyte differential count, assessed before onset of treatment (t_0) and after the second course of sunitinib (t_2). Blood counts were examined in EDTA-anticoagulated venous blood samples using a Sysmex XN 1000 hematology analyzer (Sysmex Corp; Kobe, Japan).

Based on the platelet, neutrophil, lymphocyte, and monocyte counts, we calculated the following ratios: neutrophil-to-lymphocyte (NLR), platelet-to-lymphocyte (PLR), and lymphocyte-to-monocyte (LMR). Follow-up data included the length of treatment with sunitinib, the cause of discontinuation, and the date of progression or death.

2.2 Statistical Elaboration

Medians, with lower (Q1) and upper (Q3) quartiles or means ±SD, were reported for quantitative variables. Data distribution was evaluated with the Shapiro–Wilk test. Nominal data consisted of a number or percentage of patients. Differences among patients with regression, stable disease, and disease progression were evaluated with the Kruskal–Wallis ANOVA. Progression-free survival was calculated from onset of sunitinib treatment and at the moment when progression or death occurred or was censored at the last date of follow-up in case of patients who were lost to follow-up or at study end in case of patients who remained in observation. Overall survival was calculated from onset of sunitinib treatment to death from any cause or was censored at the last date of follow-up in case of patients who were lost from the follow-up or at study. Progression-free and overall survivals were estimated using the Kaplan–Meier method and compared with the log-rank test. Associations between results of radiologic assessment after the second course of sunitinib and blood counts

are assessed at the start of the study (t_0 = Day 0) and after the second treatment course with sunitinib (t_2 = Day 85) as predictors, and progression-free survival and overall survival as outcome variables were analyzed with univariate and multiple Cox proportional hazard models. The Cox models that used the data acquired at t_2 as the predictors of progression-free survival excluded patients who received less than three courses of sunitinib treatment. Multiple Cox models included predictors that were significant in simple analysis, but strongly intercorrelated predictors (e.g., neutrophil count and NLR calculated the same time point) were not included in the single model. Details of multiple Cox models are reported in the text of the Results section and in the relevant tables. The tests were two-tailed, and results were considered significant at $p < 0.05$; calculations were performed using Statistica 12 (StatSoft Inc. Tulsa, OK).

3 Results

During the study time, we identified 131 mRCC patients treated with sunitinib as the first-line treatment. The results of a radiological examination after the second course of sunitinib treatment were available for 126 out of the 131 patients (Table 1). In 16 (13%) patients, the examination revealed a progressive disease (PD). Complete response (CR) was detected in 5 (4%) patients and partial response (PR) in 42 (33%) patients. The majority of 63 (50%) patients had a stable disease (SD) after 2 cycles of first-line sunitinib treatment. Patients with early progression, stable disease, and regression did not differ in terms of gender, age, BMI, Fuhrman score, or RCC stage at diagnosis. The MSKCC prognostic score tended to be higher in patients with PD and SD than in those with regression, but the difference failed to reach statistical significance. The IMDC score was significantly higher in patients with SD and PD (Table 1). In the 126 patients, the median observation time (lower; upper quartile) was 23 (13; 36) months, with a range of 3–70 months. The median number of treatment courses with first-line sunitinib was 9 (6; 16), with a range of 2–50. Progression-free survival for the whole group was 15 (8; 33) months. The median overall survival was 31 (17; not reached) months.

Cell blood counts with leukocyte differentials were available for 121 patients at the start of sunitinib treatment and for 110 patients after the second course of sunitinib. In 106 patients, the counts were available at onset of treatment and after the second course of treatment. After two courses of sunitinib, we observed a significant decrease in blood hemoglobin, platelet, neutrophil, lymphocyte, and monocyte counts. Moreover, NLR values were significantly lower, while there were no significant changes in PLR and LMR (Table 2).

Except for the blood hemoglobin level at onset of treatment, no other cell blood parameters at t_0 significantly associated with the results of the first radiological examination, i.e., with CR/PR, SD, or PD (Fig. 1). Consequently, NLR, PLR, and LMR values did not differ significantly across patients with CR/PR and SD and PD (Fig. 2). Changes, i.e., the absolute difference between the initial values and the values after two courses of treatment with sunitinib, in the blood hemoglobin ($p = 0.70$), platelet ($p = 0.20$), neutrophil ($p = 0.60$), lymphocyte ($p = 0.70$), and monocyte ($p = 0.50$) counts, as well as in NLR ($p = 0.60$), PLR ($p = 0.07$), and LMR ($p = 0.80$), did not differ across the patient groups.

Treatment was discontinued in 16 patients with PD after the second course of sunitinib. We thus restricted further progression-free survival analysis to the 110 patients who received at least three courses of sunitinib. The median observation time (lower; upper quartile) in this group was 25 (14; 38) months, with a range of 6–70 months. In 60 (55%) patients, treatment was stopped due to progression, and in 6 (5%) it was stopped due to toxicity. Nine (8%) patients were lost from the observation and 35 (32%) remained in treatment with first-line sunitinib at end of study. The median progression-free survival in this last group was 18 (10; 42) months.

There were no associations between both MSKCC and IMDC score and progression-free survival, but a high Fuhrman grade was a

Table 1 Patients' characteristics at onset of first-line treatment with sunitinib. Patients are grouped according to their response to treatment, assessed radiologically after the second course of sunitinib

Characteristics	All mRCC patients ($n = 126$)	Response to treatment			p-value
		CR or PR ($n = 47$)	SD ($n = 63$)	PD ($n = 16$)	
Age ± SD; years	63.1 ± 10.1	63.8 ± 9.6	62.1 ± 10.7	65.1 ± 9.3	0.500
Male gender; *n* (%)	80 (63)	31 (66)	40 (63)	9 (56)	0.800
BMI ± SD; kg/m^2	28.2 ± 4.5	27.9 ± 4.5	28.5 ± 4.9	27.4 ± 0.2	0.700
Fuhrman	48 (38)	16 (34)	28 (44)	4 (25)	0.040*
Grades 1–2; *n* (%)					
Grades 3–4; *n* (%)	44 (35)	11 (23)	26 (41)	7 (44)	
Not known	34 (27)	20 (43)	9 (14)	5 (31)	
Time from diagnosis to systemic treatment <1 year; *n* (%)	65 (52)	24 (51)	33 (52)	8 (50)	1.0
Stage IV RCC at diagnosis; n (%)	44 (35)	16 (34)	23(36)	5 (31)	1.0
ECOG performance score	72 (57)	29 (62)	36 (57)	7 (44)	0.800
0; *n* (%)					
1; *n* (%)	49 (39)	16 (34)	25 (40)	8 (50)	
2; *n* (%)	5 (4)	2 (4)	2 (3)	1 (6)	
Median Karnofsky performance scale (Q1; Q3); %	90 (80; 100)	90 (80;100)	90 (80; 100)	85 (80; 90)	0.300
MSKCC prognosis	44 (35)	21 (45)	20 (32)	3 (19)	0.100
Good; *n* (%)					
Intermediate; *n* (%)	82 (65)	26 (55)	43 (68)	13 (81)	
IMDC score	82 (65)	36 (77)	39 (62)	7 (44)	0.028*
Favorable; *n* (%)					
Intermediate; *n* (%)	36 (29)	7 (15)	21 (33)	8 (50)	
Poor; *n* (%)	4 (3)	3 (6)	1 (2)	0	
Not known	4 (3)	1 (2)	2 (3)	1 (6)	
Metastases	83 (66)	29 (62)	44 (70)	10 (62)	0.600
Lung; *n* (%)	18 (14)	5 (11)	9 (14)	4 (25)	0.400
Liver; *n* (%)	35 (28)	11 (23)	18 (29)	6 (38)	0.600
Bone; *n* (%)	68 (54)	23 (50)	34 (54)	11 (69)	0.400
Other sites; *n* (%)					
1 site, n (%)	37 (29)	14 (30)	20 (32)	3 (19)	0.700
2 sites, n (%)	52 (41)	21 (45)	25 (40)	6 (37)	
3 or more sites, n (%)	37 (29)	12 (26)	18 (29)	7 (44)	

BMI body mass index, *RCC* renal cell carcinoma, *ECOG* Eastern Cooperative Oncology Group performance score, *MSKCC* Memorial Sloan Kettering Cancer Center, *IMDC* International Metastatic Renal Cell Carcinoma Database Consortium

*p-value in chi-squared test exclusive of missing data; Q1; Q3, lower and upper quartiles

negative predictor of progression-free survival. SD after the second course of treatment associated with a significantly shorter progression-free survival than that in case of CR/PR (Fig. 3a). The lymphocyte count, NLR, and PLR at t_0 were shown to be significant predictors of progression-free survival. The neutrophil, lymphocyte, and monocyte counts and the NLR, PLR, and LMR values at t_2 were also identified as significant predictors of progression-free survival. The SD and NLR at t_2 were independent predictors of progression-free survival in multiple regressions (Table 3). Moreover, NLR categories (tertiles) were associated with progression-free survival independently of the result of a radiological examination (Fig. 3;

Table 2 Hemoglobin, blood cell counts, and blood cell ratios at onset of treatment and after the second course of sunitinib treatment

Variable	At start of treatment ($n = 121$)	After second course ($n = 110$)	p-value
Hemoglobin; g/dl	13.6 ± 1.7	12.8 ± 1.6	<0.001
Platelets; x10^3/μl	229 (190; 275)	191 (168; 223)	<0.001
Neutrophils; x10^3/μl	4.32 (3.51; 5.18)	2.23 (1.70; 3.00)	<0.001
Lymphocytes; x10^3/μl	2.03 (1.62; 2.63)	1.80 (1.39; 2.15)	<0.001
Monocytes; x10^3/μl	0.66 (0.53; 0.84)	0.56 (0.40; 0.73)	<0.001
NLR	2.13 (1.56; 2.83)	1.30 (0.96; 1.80)	<0.001
PLR	113 (90; 160)	111 (78; 151)	0.08
LMR	3.17 (2.22; 3.93)	3.08 (2.32; 4.40)	0.10

Data are means ±SD for hemoglobin and medians (lower quartile; upper quartile) for other variables; t-test values for paired variables for hemoglobin and Wilcoxon test values for other variables were calculated for 106 patients whose data were available both at onset and after the second course of sunitinib

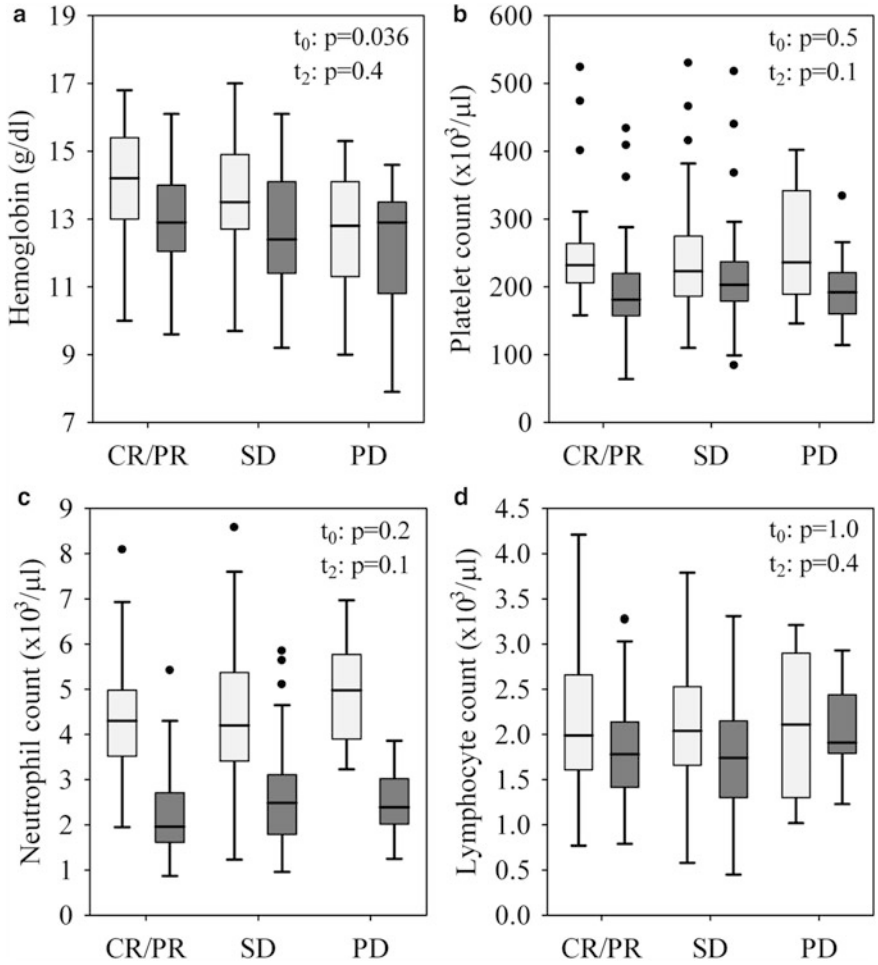

Fig. 1 Results of blood counts: hemoglobin (**a**), platelet (**b**), neutrophil (**c**), and lymphocyte (**d**) at onset of sunitinib treatment (light bars, t_0) and after the second treatment course (dark bars; t_2) in patients with complete or partial response (CR/PR), stable disease (SD), or progressive disease (PD) as assessed radiologically after the second treatment course. Data are shown as median, interquartile range (box), non-outlier range (whiskers), and outliers (points); p-values are given for the difference between CR/PR, SD, and PD groups at onset (t_0) and after two treatment courses with sunitinib (t_2)

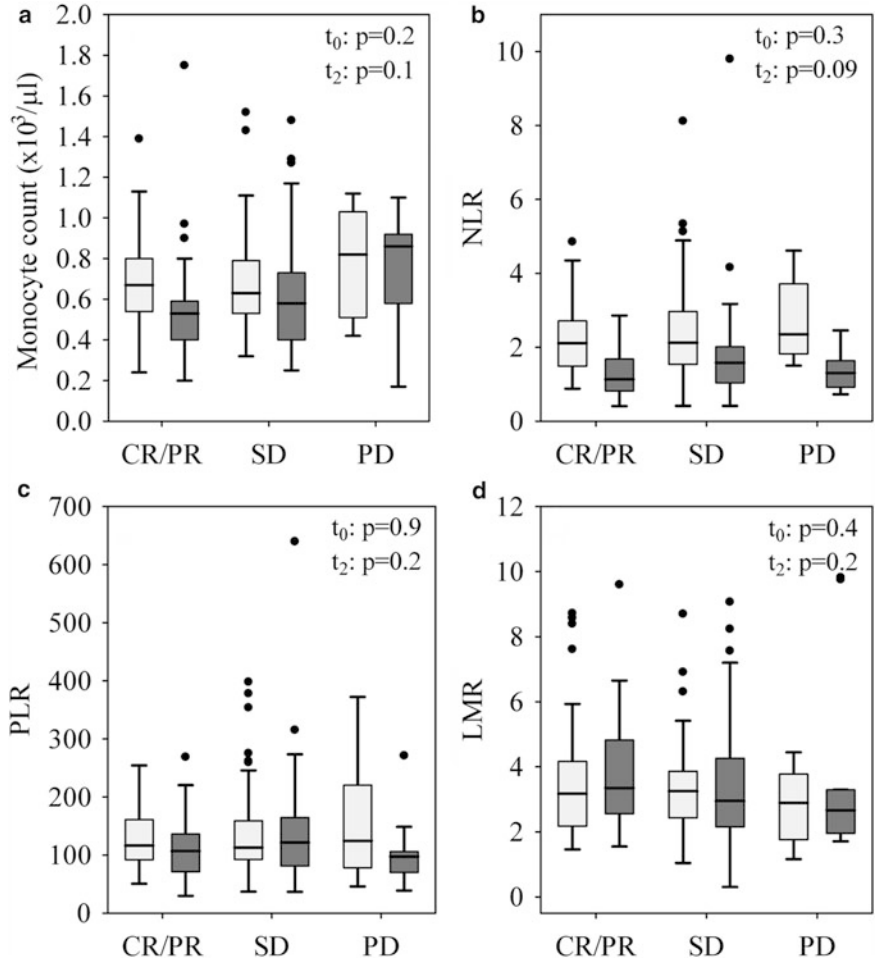

Fig. 2 Results of monocyte counts (**a**) and ratios: neutrophil-to-lymphocyte (NLR) (**b**), platelet-to-lymphocyte (PLR) (**c**), and lymphocyte-to-monocyte (LMR) (**d**) at onset of sunitinib treatment (light bars; t_0) and after the second treatment course (dark bars; t_2) in patients with complete or partial response (CR/PR), stable disease (SD), or progressive disease (PD) as assessed radiologically after the second treatment course. Data are shown as median, interquartile range (box), non-outlier range (whiskers), and outliers (points); *p*-values were given for the difference between CR/PR, SD, and PD groups at onset (t_0) and after two treatment courses with sunitinib treatment (t_2)

Table 4). Patients whose CR/PR and NLR at t_2 were in the lower or middle tertiles had the longest progression-free survival (Fig. 3c).

As expected, both MSKCC and IMDC scores were significant predictors of overall survival (Table 5). The results of a radiological examination after the second treatment course significantly associated with overall survival, with significantly shorter survival times in patients with PD at t_2 (Fig. 4a). The hemoglobin, neutrophil, and monocyte counts and NLR, PLR, and LMR at onset of sunitinib treatment were significant predictors of overall survival in simple analysis. However, only the LMR was a predictor of overall survival, independent of the IMDC score. Moreover, hemoglobin, NLR, and PLR, assessed after the second course of treatment, significantly predicted the overall survival; NLR and PLR were the predictors independent of the IMDC score. We performed multiple analyses, including the IMDC-independent predictors. In this analysis, PD and NLR at t_2 were identified as independent predictors of overall survival (Table 5). Since NLR and PLR were strongly correlated, we could not include

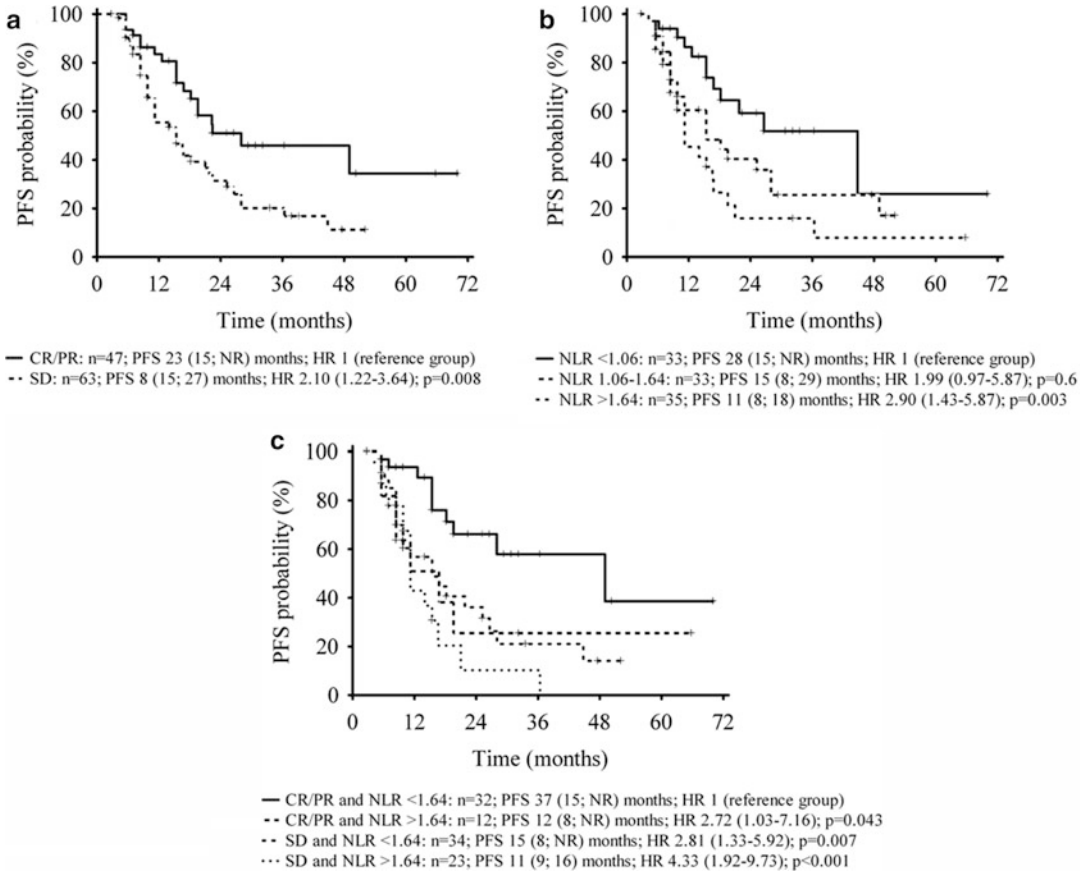

Fig. 3 Progression-free survival (PFS) of mRCC patients with respect to the results of radiological examination after the second treatment course with sunitinib (**a**) and neutrophil-to-lymphocyte ratio (NLR) after the second treatment course with sunitinib (**b**) and in categories based on both radiological examination and NLR values (**c**). The analysis included patients who continued treatment after the second course with sunitinib, i.e., patients with disease progression at t_2 were excluded. The number of patients, medians (lower-upper quartiles) of overall survival, and hazard ratios (HR) with 95% confidence intervals were given; *p*-values in unadjusted Cox regression are shown below the graphs; *CR* complete response, *PR* partial response, *SD* stable disease, *NR* not reached

them both in a regression model. When PLR was included in a multiple model, instead of NLR, it was not a significant predictor of overall survival (data not shown). Further, we categorized NLR at t_2 by tertiles and found that NLR in the upper tertile significantly associated with a shorter overall survival (Fig. 4b), independent of IMDC scores and PD at t_2 (Table 6). As shown in Fig. 4c, patients with CR/PR and SD and NLR in the lower or middle tertile had a longer overall survival than those with CR/PR and SD and NLR in the upper tertile. The patients with early radiological progression had a shorter overall survival.

4 Discussion

In the present study, multiple regression analysis confirmed that attaining complete or partial remission at the time of the first radiological evaluation was associated with a longer progression-free and overall survival. On the other side, when disease progression was identified at the time of the first radiologic evaluation after two courses of sunitinib treatment, overall survival was distinctly shorter. In previous sunitinib studies, the objective response rate among mRCC patients has been 31%, with the

Table 3 Predictors of progression-free survival in simple Cox regression, after adjustment for the IMDC score, and in multiple Cox regression. The analysis included patients with complete remission/partial remission (CR/PR) or stable disease (SD) after second course of sunitinib treatment, i.e., those who received at least three courses of sunitinib. The cell blood counts were assessed at onset of treatment (t_0) and after the second course of treatment with sunitinib (t_2)

	Hazard ratio (95% confidence interval) for progression; *p*-value		
Predictor variable	Simple cox regression	Adjusted for the results of radiological assessment at t_2 (SD vs. CR/PR)	Multiple cox regression
Intermediate MSKCC	1.30 (0.76–2.22); $p = 0.300$	–	–
Intermediate/poor IMDC	1.55 (0.89–2.69); $p = 0.100$	–	–
Fuhrman grades 3–4	1.79 (1.01–3.19); $p = 0.047$	1.76 (0.98–3.13); $p = 0.060$	–
SD at t_2	2.10 (1.22–3.64); $p = 0.008$	–	2.27 (1.24–4.15); $p = 0.008$
Hemoglobin at t_0; *per* 1 g/dl	0.89 (0.75–1.04); $p = 0.100$	–	–
Platelets at t_0; *per* 10×10^3/μl	1.00 (0.97–1.04); $p = 0.800$	–	–
Neutrophils at t_0; *per* 1×10^3/μl	1.15 (0.95–1.39); $p = 0.100$	–	–
Lymphocytes at t_0; *per* 1×10^3/μl	0.66 (0.44–0.98); $p = 0.038$	0.69 (0.46–1.02); $p = 0.070$	–
Monocytes at t_0; *per* 1×10^3/μl	1.68 (0.56–5.02); $p = 0.400$	–	–
NLR at t_0; *per* 1	1.22 (1.04–1.44); $p = 0.017$	1.17 (0.99–1.37); $p = 0.070$	–
PLR at t_0; *per* 10	1.03 (1.00–1.06); $p = 0.08$	–	–
LMR at t_0; *per* 1	0.76 (0.61–0.95); $p = 0.014$	0.75 (0.60–0.94); $p = 0.013$	0.81 (0.63–1.04); $p = 0.100$
Hemoglobin at t_2; *per* 1 g/dl	0.96 (0.80–1.14); $p = 0.600$	–	–
Platelets at t_2; *per* 10×10^3/μl	1.00 (0.96–1.03); $p = 0.800$	–	–
Neutrophils at t_2; *per* 1×10^3/μl	1.31 (1.01–1.69); $p = 0.041$	1.22 (0.94–1.59); p = 0.100	–
Lymphocytes at t_2; *per* 1×10^3/μl	0.63 (0.40–0.97); $p = 0.036$	0.63 (0.42–0.96); $p = 0.033$	–
Monocytes at t_2; *per* 1×10^3/μl	2.52 (1.05–6.08); $p = 0.040$	2.13 (0.86–5.29); $p = 0.100$	–
NLR at t_2; *per* 1	1.42 (1.18–1.70); $p < 0.001$	1.34 (1.11–1.63); $p = 0.002$	1.28 (1.02–1.61); $p = 0.037$
PLR at t_2; *per* 10	1.04 (1.01–1.08); $p = 0.006$	1.04 (1.00–1.07); $p = 0.030$	–
LMR at t_2; *per* 1	0.73 (0.59–0.91); $p = 0.006$	0.77 (0.62–0.94); $p = 0.012$	–

MSKCC Memorial Sloan Kettering Cancer Center, *IMDC* International Metastatic Renal Cell Carcinoma Database Consortium, *NLR* neutrophil-to-lymphocyte ratio, *PLR* platelet-to-lymphocyte ratio, *LMR* lymphocyte-to-monocyte ratio

best response being evaluated, progression-free survival was 10.1 months (Motzer et al. 2007), and overall survival was 26 months (Motzer et al. 2009). Relative to those values, the present results appeared better as we noticed the objective response rate of 37%, evaluated after the second course of sunitinib treatment, the median progression-free survival of 15 months, and the

Table 4 Cox regression model based on the results of radiological assessment and neutrophil-to-lymphocyte ratio (NLR) tertiles after the second course of sunitinib treatment (at t_2) to predict progression-free survival in metastatic renal cell carcinoma (mRCC) patients treated with sunitinib who received at least three courses of treatment, i.e., excluding patients with disease progression at t_2

Predictor variable	Hazard ratio (95% confidence interval) for progression	*p*
CR/PR at t_2	1 (reference group)	
SD at t_2	2.31 (1.29–4.14)	0.005
NLR at $t_2 \leq 1.06$	1 (reference group)	
NLR at t_2 between 1.06 and 1.64	2.06 (1.01–4.22)	0.047
NLR at $t_2 > 1.64$	2.70 (1.33–5.48)	0.006

CR/PR complete remission/partial remission, *SD* stable disease

Table 5 Predictors of overall survival in simple Cox regression, after adjustment for the IMDC score, and in multiple Cox regression. The analysis included all patients with available data, regardless of the results of radiological assessment after the second course of sunitinib treatment. Cell blood counts were assessed at onset of treatment (t_0) and after the second course of treatment with sunitinib (t_2)

	Hazard ratio (95% confidence interval) for death; *p*-value		
Predictor variable	Simple Cox regression	Adjusted for IMDC	Multiple Cox regression
Intermediate MSKCC	2.60 (1.43–4.71); $p = 0.002$	–	–
Intermediate/poor IMDC	2.98 (1.77–5.01); $p < 0.001$	–	2.59 (1.47–4.56); $p < 0.001$
PD at t_2	3.29 (1.81–6.00); $p < 0.001$	2.72 (1.45–5.11); $p = 0.002$	5.86 (2.57–13.37); $p < 0.001$
Hemoglobin at t_0; *per* 1 g/dl	0.78 (0.66–0.91); $p = 0.002$	0.86 (0.72–1.03); $p = 0.100$	–
Platelets at t_0; *per* 10×10^3/μl	1.02 (0.98–1.05); p = 0.300	–	–
Neutrophils at t_0; *per* 1×10^3/μl	1.21 (1.02–1.44); $p = 0.032$	1.09 (0.91–1.30); $p = 0.300$	–
Lymphocytes at t_0; *per* 1×10^3/μl	0.79 (0.53–1.18); $p = 0.300$	–	–
Monocytes at t_0; *per* 1×10^3/μl	3.01 (1.08–8.34); $p = 0.034$	2.25 (0.80–6.33); $p = 0.100$	–
NLR at t_0; *per* 1	1.26 (1.06–1.50); $p = 0.008$	1.13 (0.94–1.35); $p = 0.200$	–
PLR at t_0; *per* 10	1.03 (1.00–1.07); $p = 0.024$	1.01 (0.98–1.05); $p = 0.400$	–
LMR at t_0; *per* 1	0.74 (0.58–0.93); $p = 0.010$	0.79 (0.63–0.98); $p = 0.029$	0.89 (0.71–1.12); $p = 0.300$
Hemoglobin at t_2; *per* 1 g/dl	0.80 (0.67–0.98); $p = 0.015$	0.87 (0.72–1.06); $p = 0.200$	–
Platelets at t_2; *per* 10×10^3/μl	1.01 (0.97–1.04); $p = 0.800$	–	–
Neutrophils at t_2; *per* 1×10^3/μl	1.21 (0.95–1.55); $p = 0.100$	–	–
Lymphocytes at t_2; *per* 1×10^3/μl	0.77 (0.50–1.18); $p = 0.200$	–	–
Monocytes at t_2; per 1×10^3/μl	2.09 (0.86–5.14); $p = 0.100$	–	–
NLR at t_2; *per* 1	1.27 (1.07–1.51); $p = 0.007$	1.28 (1.05–1.55); $p = 0.013$	1.28 (1.02–1.60); $p = 0.035$
PLR at t_2; *per* 10	1.04 (1.01–1.06); $p = 0.010$	1.04 (1.01–1.07); $p = 0.013$	–
LMR at t_2, *per* 1	0.90 (0.75–1.07); $p = 0.200$	0.91 (0.77–1.08); p = 0.300	–

MSKCC Memorial Sloan Kettering Cancer Center, *IMDC* International Metastatic Renal Cell Carcinoma Database Consortium, *PD* progressive disease, *NLR* neutrophil-to-lymphocyte ratio, *PLR* platelet-to-lymphocyte ratio, *LMR* lymphocyte-to-monocyte ratio

median overall survival of 31 months. A greater median progression-free survival may reflect the fact that in typical medical practice, disease progression tends to be recognized later than in clinical trials. On the other side, a greater overall survival may reflect the introduction of new therapies after disease progression during initial treatment.

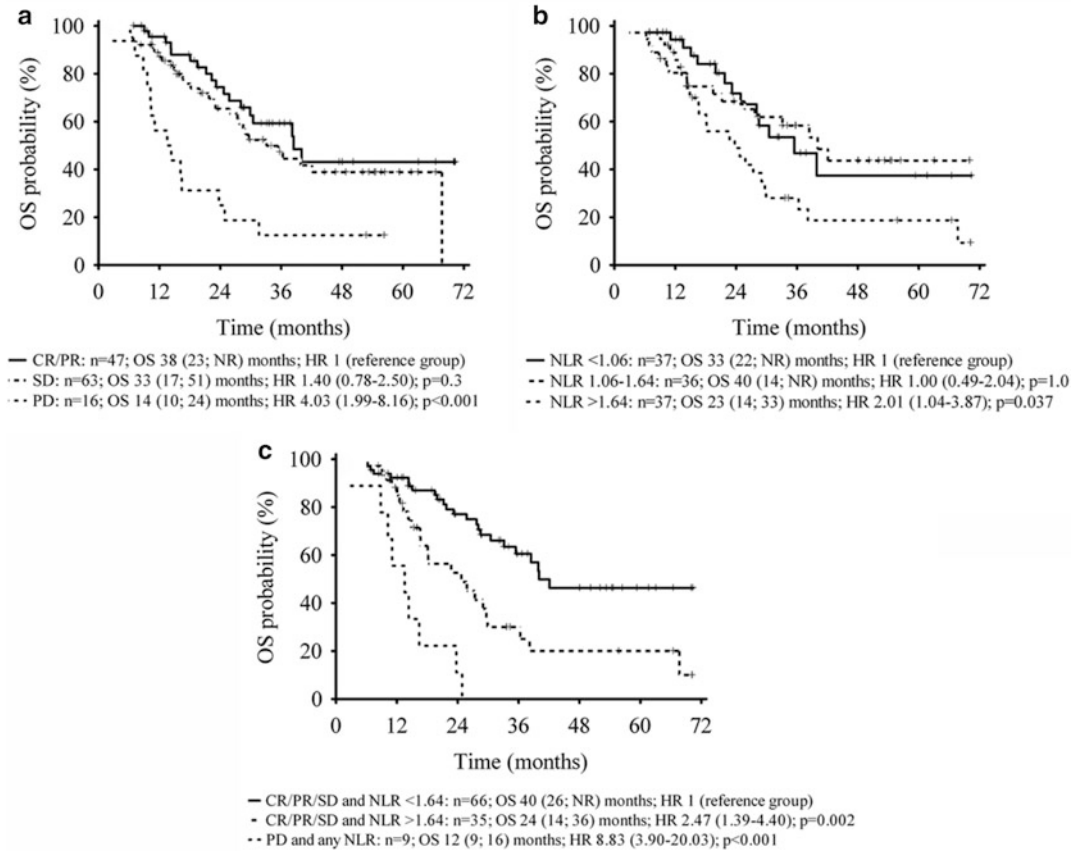

Fig. 4 Overall survival (OS) of mRCC patients with respect to the results of radiological examination after the second treatment course with sunitinib (**a**) and neutrophil-to-lymphocyte ratio (NLR) after the second treatment course with sunitinib (**b**) and in categories based on both radiological examination and NLR values (**c**). The number of patients, medians (lower-upper quartiles) of OS and hazard ratios (HR) with 95% confidence interval, and *p*-values in unadjusted Cox regression were given below the graphs; *CR*, complete response; *PR*, partial response; *SD*, stable disease; *NR*, not reached

Table 6 Multiple Cox regression model based on the results of radiological assessment and neutrophil-to-lymphocyte ratio (NLR) tertiles after the second course of sunitinib treatment (at t_2) to predict overall survival in metastatic renal cell carcinoma (mRCC) patients treated with sunitinib

Predictor variable	Hazard ratio (95% confidence interval) for death	*p*-value
Favorable IMDC score	1 (reference group)	
Intermediate/poor IMDC score	2.52 (1.42–4.48)	0.002
CR/PR/SD at t_2	1 (reference group)	
PD at t_2	5.70 (2.49–13.02)	<0.001
NLR at $t_2 \leq 1.06$	1 (reference group)	
NLR at t_2 between 1.06 and 1.64	1.23 (0.57–2.69)	0.600
NLR at $t_2 > 1.64$	2.05 (1.04–4.05)	0.039

IMDC International Metastatic Renal Cell Carcinoma Database Consortium score, *CR/PR* complete remission/partial remission, *SD* stable disease, *PD* progressive disease

Cancer patients' response to treatment is affected by both tumor characteristics and host response to tumor growth. This response is a result of a balance between immune response and cancer-related inflammation (Grivennikov et al. 2010). Tumor cells stimulate expression of inflammatory mediators that promote tumor growth, progression, angiogenesis, invasion, and metastases. Cancer-related inflammation is reflected neutrophilia, monocytosis, thrombocytosis, and lymphopenia in peripheral blood cell counts. Neutrophils display the immunosuppressive potential by releasing reactive oxygen and nitric oxide species, which may mitigate the tumor response mediated by the patient's T cells (Uribe–Querol and Rosales 2015; Kowalczyk et al. 2006). Elevated NLR suggests more advanced disease and progression, and it is a sign of imbalance in the immune response, which limits the body's normal antitumor reaction. The present study confirmed the significance of NLR in the initial prognostic assessment in mRCC patients as high NLR values were associated with shorter progression-free and overall survival.

The evaluation 3 months after onset of treatment showed that the patients' prognosis depended, to a substantial extent, on the results of radiological examination. The NLR at this time point was found to be an independent, statistically significant predictor of the patients' progression-free and overall survival. Keizman et al. (2012) have found an association between NLR and prognosis in mRCC patients, showing an adverse relationship between NLR and the objective response rate and progression-free and overall survival. Those authors have also assessed the relationship between treatment outcomes and NLR after the first course of sunitinib treatment but failed to find any significant association in this case. The difference between those findings and the present ones could be explained by the time at which the evaluations took place, 6 weeks vs. 12 weeks from onset of treatment, respectively. The evaluation at 12 weeks seems justified by the time to response, which has a median of 10.6 weeks in the first-line treatment of mRCC patients (Molina et al. 2014). Tannir et al. (2017) have analyzed the so-called long value responders, defined as patients with progression-free survival of 18 months or more, and found that both the initial NLR and after the first course of sunitinib treatment are predictors of the objective response rate and progression-free and overall survival. Likewise, Templeton et al. (2016) have found an association between NLR after 6 ± 2 weeks from onset of sunitinib treatment and the objective response rate and progression-free and overall survival. Those studies, however, have not evaluated whether NLR would be an independent predictor of radiological response to treatment, which was a key goal of the present study.

The monocytes, another type of inflammatory cells, particularly CD16+ cells, play a role in the antitumor response. However, circulating CD16+ monocytes promote tumor growth in some diseases. Monocytes express angiogenic chemokines, such as CXCL3, and secrete angiogenic factors such as interleukin-8, vascular endothelial factor (VGEF), and fibroblast growth factor, which may induce tumor angiogenesis and progression (Han et al. 2017; Pastewka et al. 2010). In the present study, we noticed an adverse association between LMR at t_0 and t_2 and progression-free survival, but no association between LMR and overall survival in multiple regression analysis.

Thrombocytosis in cancer patients is a common abnormality. Myeloid-derived supressor cells induce platelet activation. In some patients with thrombocytosis, elevated serum levels of thrombopoietin have been observed. Platelets and platelet-derived microparticles are the main transporter of proangiogenic factors, such as VEGF, microRNA, and lipids, which contribute to angiogenesis. Since tumor growth requires the formation of new blood vessels to provide nutrition and oxygen, increased platelet count may contribute to tumor growth and progression (Zhao et al. 2018; Wojtukiewicz et al. 2017). In the present study, PLR adversely associated with

overall survival but only when elaborated independently of the IMDC scale. That might be explicable by the fact that platelet count is just one of the factors this scale evaluates. The PLR evaluated at 12 weeks after onset of treatment had an independent predictive value, but multiple regression analysis did not allow for the simultaneous evaluation of NLR and PLR, since both are associated with the inflammatory process. In multiple regressions, PLR lost its predictive significance in relation to overall survival, while NLR remained its significant predictor.

In conclusion, this study shows that the neutrophil-to-lymphocyte ratio evaluated after the second treatment course with sunitinib provides the independent information on the expected progression-free and overall survival. The longest progression-free and overall survival rates were achieved by patients who had both objective response according to RECIST 1.1 and the neutrophil-to-lymphocyte ratio lower than 1.64.

Conflicts of Interest JK has received research grant from Novartis and travel grants and speakers' honoraria from Pfizer, Bayer, Novartis, BMS, and IPSEN. PW has received speakers' honoraria and travel grants from Pfizer, Bayer, Novartis, and IPSEN. The other authors declare no conflicts of interest in relation to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Maria Sklodowska-Curie Memorial Cancer Center and the Institute of Oncology (permission 38/2018).

Informed Consent For this retrospective type of study, formal consent from individual patients was not required.

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Plasminogen Activator Inhibitor Type 1 in Blood at Onset of Chemotherapy Unfavorably Affects Survival in Primary Ovarian Cancer

Justyna Teliga-Czajkowska, Jacek Sienko, Katarzyna Jalinik, Paweł Derlatka, Anna Danska-Bidzinska, and Krzysztof Czajkowski

Abstract

Plasminogen activator inhibitor type 1 (PAI-1) belongs to the family of the plasminogen activator system. PAI-1 stimulates fibrinolysis and also promotes tumor progression. The aim of this study was to evaluate the prognostic value of blood plasma PAI-1 content in patients with epithelial ovarian cancer who start the first-line chemotherapy. PAI-1 content was measured in the blood of 61 patients with epithelial ovarian cancer at onset of first-line chemotherapy. The patients were further stratified into the low PAI-1 group (≤20 ng/mL; 33 patients) and the high PAI-1 group (>20 ng/mL; 28 patients). We found that the greater plasma PAI-1 content was associated with a significantly lower probability of a 5-year-long survival compared to that when PAI-I content was lower (45.5% vs. 69.5%, respectively; $p = 0.04$). However, the risk of cancer recurrence within 5 years failed to differ appreciably. A multivariate analysis revealed that the lower PAI-1 plasma content was an independent factor of longer overall survival (death risk ratio of 0.36, 95%CI = 0.16–0.79; $p < 0.01$). We conclude that PAI-1 is yet another biomarker of survival in patients with ovarian cancer.

Keywords

Biomarker · Cancer-free survival · Epithelial ovarian cancer · Fibrinolysis · Overall survival · PAI-1 · Tumor progression

J. Teliga-Czajkowska
Department of Obstetrics and Gynecology Didactics, Medical University of Warsaw, Warsaw, Poland

J. Sienko (✉), P. Derlatka, A. Danska-Bidzinska, and K. Czajkowski
Second Department of Obstetrics and Gynecology, Medical University of Warsaw, Warsaw, Poland
e-mail: jacek.sienko@wum.edu.pl

K. Jalinik
Department of Gynecologic Oncology, The Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland

1 Introduction

Malignancy causes a derangement of body hemostasis which predisposes cancer patients to both thrombosis and hemorrhage (Falanga et al. 2013). Malignant tumor cells interact with vascular endothelium, leading, in turn, to the activation of a coagulation cascade. The prothrombotic environment increases the risk of thrombotic events and favors tumor growth and metastasis (Falanga et al. 2015). Fibrin provides a scaffold for cancer cell anchorage and protects them from the recognition and

destruction by the immune system. The activated factors VII and X and thrombin promote invasive growth via the induction of receptor-mediated intracellular signals (Falanga and Marchetti 2018; Falanga et al. 2009).

Epithelial ovarian cancer (EOC) is the leading cause of cancer-related deaths among females in the developed countries (Siegel et al. 2018). Approximately 70% of patients are diagnosed at an advanced stage when a 5-year survival rate is a scarce 10–20% (Coleman et al. 2011). The elevated fibrinogen and D-dimer levels are associated with worse prognosis in epithelial ovarian cancer (Man et al. 2015) and in primary serous cancer (Liu et al. 2015). Enhanced response to hypercoagulation, namely, fibrinolysis, also plays a role in tumor invasion and metastasis. The plasminogen activator system includes urokinase-type plasminogen (uPA), its receptor (uPAR), tissue PA (t-PA), and type 1 and 2 plasminogen inhibitors (PAI-1 and PAI-2) (Mengele et al. 2010). PAI-1 controls both cell surface expression and internalization of uPA-uPAR. The binding of PAI-1 forms a trimetric complex PAI-1-uPA-uPAR which is recognized by lipoprotein-related protein and internalized for endocytosis. PAI-1 stimulates the invasion of tumor cells and the development of metastases by modulated cell adhesion, stimulation of cell proliferation, and inhibition of excess degradation of extracellular matrix (Jaiswal et al. 2018; Deng et al. 2002). The PAI-1-uPA-uPAR system is also involved in VEGF-induced angiogenesis, which contributes to tumor progression. A high level of PAI-1 turns off plasminogen activator-dependent proteolysis (Prager et al. 2004; Devy et al. 2002). PAI-1 is known as an acute phase reactant, and as such it increases in patients with inflammatory diseases not associated with cancer (Juhan-Vague et al. 1985). However, the increase in PAI-1 content is greater in malignancies compared with inflammatory diseases (Ho et al. 1999). Konecny et al. (2001) have measured PAI-1 content in EOC tumor samples and found that its elevated level is associated with shorter progression-free and overall survival, but does not constitute an independent factor of prognosis. Detection of biomarkers obtained from tumor tissue during surgery is important but impractical. The exact prognosis is usually made at onset of adjuvant chemotherapy when fresh tissue specimens are yet unavailable. Attempts have been undertaken to sort out a coagulation laboratory test which would be useful in a prompt evaluation of cancer prognosis. Plasma PAI-1 content seems to have offered promise to this end (Havrilesky et al. 2008). Therefore, this study seeks to define a prognostic value of the plasma content of PAI-1 in patients with EOC at onset of the first-line chemotherapy.

2 Methods

2.1 Patients and Samples

The study was conducted in patients with EOC in the Princess Anna Mazowiecka Hospital of Medical University of Warsaw in Poland between 2011 and 2018. There were 61 patients, who had previously undergone surgery, enrolled into the study at onset of the first-line chemotherapy. All the patients received adjuvant chemotherapy that included six cycles of intravenous paclitaxel plus carboplatin. The clinico-pathological data were obtained from medical records. Blood samples were taken at onset of chemotherapy for PAI-1 and for other coagulation factors including fibrinogen, D-dimer, and antithrombin III (AT III). Prothrombin time (PT), activated partial thromboplastin time (APTT), and international normalized ratio (INR) were assessed. The IMUBIND® Plasma PAI-1 ELISA kit (Sekisui Diagnostics LLC; Lexington, MA) was used for the quantitative measurement of human PAI-1 antigen in the plasma. Effects of chemotherapy were assessed according to the response evaluation criteria in solid tumors (RECIST) 1.1 criteria (Eisenhauer et al. 2009). Overall survival was defined as the time from onset of treatment until death from any cause. Disease-free survival was defined as the time from onset of treatment until disease progression or death, according to Food and Drug Administration guidance (CDER/CBER 2018). The patients were stratified into low of $\leq$20 ng/mL and high >20 ng/mL PAI-1.

The mean follow-up from the initial treatment was 57.0 ± 22.7 months (range of 2.6–87.6 months).

2.2 Statistical Elaboration

Data were presented as means ±SD. Differences between the two groups of PAI-I level were assessed with a Chi-squared test, exact Fisher's test, or Student's *t*-test. The Kaplan-Meier plots were created to estimate the survival curves, and differences in survival were compared using the log-rank test. The Cox regression model was used to ascertain the value of independent prognosis for postoperative patients with EOC. A *p*-value of <0.05 defined statistically significant differences.

3 Results

3.1 Patient Characteristics

The low PAI-1 (≤20 ng/mL) group consisted of 33 patients and the high PAI-1 (>20 ng/mL) of 28 patients. The clinico-pathological characteristics of the groups were presented in Table 1. The groups did not differ in terms of age, tumor stage and grade, and the body mass index. The results of the first-line chemotherapy are presented in Table 2. No differences between the groups were observed regarding the response to chemotherapy. Nor was there any significant difference between the groups of the lower and higher PAI-1 level when all kinds of responses to chemotherapy were taken *versus* diseases progression. The results of other coagulation parameters assessed at onset of chemotherapy were presented in Table 3.

3.2 Survival Estimation

The Kaplan-Meier plots were used to compare overall survival and disease-free survival between low and high PAI-1 patients. The patients with PAI-1 of >20 ng/mL had a significantly lower probability of a 5-year survival than those with PAI-1 of ≤20 ng/mL (45.5% vs. 69.5%, respectively; $p = 0.04$) at onset of chemotherapy (Fig. 1a), but the difference in a chance for disease-free survival within 5 years failed to reach significance (47.5% vs. 68.5%, respectively; $p = 0.17$) (Fig. 1b).

Univariate analysis showed that a high PAI-1 level, age > 60 years, advanced tumor stage, and tumor grade 3 were associated with worse overall survival (Table 4). Multivariate analysis revealed that PAI-1 was an independent marker of poor overall survival (Table 5). Tumor stage and grade were also independent predictors of overall survival.

4 Discussion

PAI-1, as an inhibitor of fibrinolysis, plays an important role in the response to activation of the coagulation system caused by different stimuli. It inhibits uPA-dependent conversion of plasminogen to plasmin leading to degradation of fibronectin, laminin, and collagen in the basement membranes and extracellular matrix (Van De Craen et al. 2012). Ample evidence has accumulated on the prognostic value of tumor-associated proteolytic factors in patients with solid malignant tumors. PAI-1 expression has been associated with a disease outcome and is an independent prognostic factor in several malignancies including breast, lung, kidney, gastrointestinal tract, and gynecological cancer (van Dam et al. 2017). Biomarkers uPA/PAI-1, as recommended by ASCO, can be evaluated in primary breast cancer to avoid unnecessary chemotherapy in patients at medium risk for recurrence (Harris et al. 2016). In endometrial cancer, a high content of PAI-1 and uPA, determined in the cytosolic fraction of endometrial carcinoma, after adjustment for well-established clinical prognostic factors, was associated with shorter progression-free survival (Steiner et al. 2008; Fredstorp-Lidebring et al. 2001). There have been a few studies reporting either poor outcome or no correlation with survival in patients with ovarian tumors overexpressing proteases such as PAI-1 (Hoffmann et al. 1999; Kuhn et al. 1999).

Table 1 Clinico-pathological characteristics of the low and high plasminogen activator inhibitor type 1 (PAI-1) groups of patients

	PAI-1 ≤ 20 ng/mL ($n = 33$)	PAI-1 > 20 ng/mL ($n = 28$)	p
Age (years)	53.3 ± 12.9	57.4 ± 10.5	0.19
BMI ≤ 25 (kg/m^2)	11 (41%)	16 (59%)	0.06
Histological type; *n* (%)			0.54
Serous	15 (45.4%)	15 (53.6%)	
Endometrioid	9 (6.1%)	1 (3.6%)	
Clear cell	7 (27.3%)	6 (21.4%)	
Mucinous	2 (21.2%)	6 (21.4%)	
Stage; n (%)			0.46
I	12 (36.4%)	6 (21.4%)	
II	3 (9.1%)	3 (10.7%)	
III	18 (54.5%)	18 (64.3%)	
IV	0 (0%)	1 (3.6%)	
Grade; *n* (%)			0.99
1	8 (24.2%)	7 (25.0%)	
2	12 (36.4%)	10 (35.7%)	
3	13 (39.4%)	11 (39.3%)	
Early cancer (FIGO I–II)	15 (45.5%)	18 (54.5%)	0.21
Advanced cancer (FIGO III–IV)	9 (32.1%)	19 (67.9%)	

Values are means ±SD or number (%) of cases in groups. *BMI* body mass index, *FIGO* staging systems of the International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d'Obstétrique). Student's *t*-test or exact Fisher's test were applied, respectively, for statistical comparisons

Table 2 Correlation between plasminogen activator inhibitor type 1 (PAI-1) plasma level and clinical results of the first-line chemotherapy in the low and high PAI-1 groups of patients

Response to treatment	PAI-1 ≤ 20 ng/mL ($n = 33$)	PAI-1 > 20 ng/mL ($n = 28$)	p
Complete remission	28 (84.9%)	23 (82.2%)	0.42
Partial remission	1 (3.0%)	2 (7.1%)	
Stabilization	0 (0%)	1 (3.6%)	
Progression	4 (12.1%)	2 (7.1%)	

Values are number (%) of cases in groups. Student's *t*-test and exact Fisher's test were applied, respectively, for statistical comparisons

Table 3 Results of coagulation tests in the low and high PAI-1 groups of patients

	PAI-1 ≤ 20 ng/mL ($n = 33$)	PAI-1 > 20 ng/mL ($n = 28$)	p
Prothrombin time (s)	11.59 ± 1.15	11.51 ± 0.86	0.79
INR	1.05 ± 0.10	1.05 ± 0.08	0.83
APTT (s)	28.38 ± 2.65	28.47 ± 3.87	0.92
D-dimer (mg/L)	3.31 ± 5.24	1.46 ± 1.71	0.06
Fibrinogen (g/L)	3.66 ± 0.81	3.46 ± 0.76	0.30
ATIII (%)	97.80 ± 12.54	105.34 ± 15.23	0.06

Values are means ±SD. *INR* international normalized ratio, *APTT* activated partial thromboplastin time, *ATIII* anti-thrombin III. Student's *t*-test was applied for statistical comparison

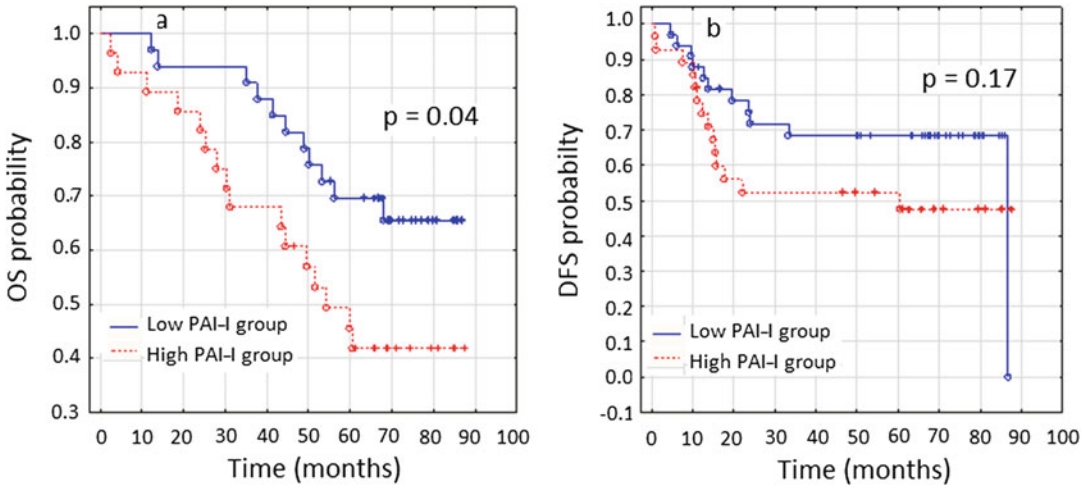

Fig. 1 Kaplan-Meier plots compared with a log-rank test: (**a**) overall survival (OS) and (**b**) disease-free survival (DFS)

Table 4 Univariate Cox regression analysis of factors associated with overall survival in patients with epithelial ovarian carcinoma

Variable	RR	95%CI	*p*
Low vs. high PAI-1	0.45	0.21–0.97	0.040
Age ≤60 years vs. >60 years	0.52	0.28–0.96	0.042
BMI ≤25 kg/m^2 vs. >25.0 kg/m^2	0.66	0.35–1.22	0.180
FIGO I–II vs. III–IV	0.09	0.03–0.29	0.00007
Grade 1 vs. grades 2–3	0.30	0.16–0.63	0.001
Serous vs. non-serous tumor	0.49	0.23–1.02	0.060

RR relative risk of death, *95%CI* 95% confidence intervals, *PAI-1*, plasminogen activator inhibitor type 1, *BMI* body mass index, *FIGO* staging systems of the International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d'Obstétrique)

Table 5 Multivariate Cox regression analysis of factors associated with overall survival in patients with epithelial ovarian carcinoma

Variable	RR	95%CI	*p*
Low vs. high PAI-1	0.36	0.16–0.79	0.010
Age ≤60 years vs. >60 years	0.62	0.27–1.41	0.250
FIGO I–II vs. III–IV	0.10	0.03–0.35	0.0003
Grade 1 vs. grades 2–3	0.27	0.12–0.62	0.002

RR relative risk of death, *95%CI* 95% confidence intervals, *PAI-1* plasminogen activator inhibitor type 1, *FIGO* staging systems of the International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d'Obstétrique)

Most investigators have determined PAI-1 and other biomarkers in tumor tissue extracts taken from the ovaries, which is not clinically convenient (Mashiko et al. 2015; Chambers et al. 1998; Wang et al. 2009). Van der Burg et al. (1996) have determined cytosolic levels of uPA and PAI-1 in 244 human ovarian tissues of different histological subtypes. Those authors have found that both uPA and PAI-1 are associated with the presence of malignancy but not with progression-free and overall survival or with other prognostic factors including the patient's age, FIGO stage, tumor grade, residual disease, and the presence of ascites. However, Hornung et al. (2004) have

failed to show the prognostic or predictive value of PAI-1 in tissue specimens of ovarian cancer.

In the present study, we set out to assess the content of PAI-1 in the blood plasma of patients suffering with epithelial ovarian cancer on the premise that this kind of assessment would be more accessible and easier and therefore more useful in everyday clinical practice. Further, tumor samples could be unavailable in patients starting chemotherapy. The investigators, who assessed the content of biomarkers in the plasma, have mostly focused on the comparison between malignant and benign changes of the ovaries (Zhang et al. 2013). Ho et al. (1999) have concluded that plasma content of PAI-1 is associated with the presence of malignant ovarian disease and with a higher stage of cancer. However, those authors have not found a significant association between the plasma and ovarian tissue content of PAI-1 and have stopped short of discussing the prognostic value of PAI-1 in either tissue. In contradistinction, in this study, we assessed PAI-1 content at onset of chemotherapy at the time when prognosis is comprehensively discussed with the patient and the decision about adjuvant treatment is made. PAI-1, if it were a prognostic biomarker, could be useful in the decision-making process at this stage of the ovarian cancer management. We found that the plasma PAI-1 content greater than 20 ng/mL at onset of chemotherapy was associated with a significantly lower probability of a 5-year survival. We could not substantiate any study in the literature that would investigate the prognostic value of PAI-1 in a similar clinical setting. Kuhn et al. (1999) have evaluated the influence of the protease PAI-1 on overall survival in patients with advanced ovarian cancer in FIGO stage IIIc in order to select patients at risk. The authors have found that PAI-1, in addition to the traditional prognostic parameters, is of prognostic significance in both univariate and multivariate analysis for overall survival. The prognostic power of PAI-1 increases with time. However, PAI-I was determined in the primary tumor tissue extract.

In the resent study, the difference in risk of recurrence within 5 years failed to differ significantly between the patients with the lower and higher PAI-1 plasma contents. That could possibly be due to a small study group. Alternatively, a longer follow-up could be needed to settle the long-term effects of PAI-1 content on patients' survival in ovarian cancer. In the aforementioned studies, progression-free survival has not been addressed (Wang et al. 2009; Kuhn et al. 1999).

Chambers et al. (1998) have found that PAI-1 is a rather poor prognostic factor for survival in epithelial ovarian carcinoma, but it turns out to be an independent prognostic marker in carcinoma stages III and IV in multivariate analysis. Nakatsuka et al. (2017) have also noticed that a strong expression of PAI-1 is associated with poor prognosis in patients with ovarian cancer. In that study, PAI-1 content is an independent predictor of reduced progression-free, but not of overall survival. Those studies have been conducted in tissue samples. In the present study, PAI-1 content in the blood plasma was an independent marker of overall survival at onset of primary chemotherapy.

5 Conclusion

Blood PAI-1 content is an independent adverse predictor of survival in patients with epithelial ovarian cancer who start the first-line chemotherapy.

Conflicts of Interest The authors declare no conflicts of interest related to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the local Bioethics Committee of Medical University of Warsaw in Poland.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Sialidase Attenuates Epidermal Growth Factor Response and Abolishes Antiproliferative Effects of Erlotinib in A549Alveolar Epithelial Cells

A. Rybak, M. Zarzecki, E. Golabiewska, A. Niechoda, and A. Holownia

Abstract

Erlotinib is a widely used, reversible tyrosine kinase inhibitor (TKI), targeting pro-proliferative signaling of epidermal growth factor receptor (EGFR). The drug is approved for the first-line treatment of patients with metastatic non-small cell lung cancer with EGFR mutations. Extracellular glycans can affect EGFR expression, dimerization, phosphorylation, and EGF binding. In this study we investigated the effects of EGF and erlotinib on the cell cycle of naive and sialidase (alpha-neuraminidase)-pretreated human A549 alveolar epithelial cells. A549 cells were labeled with propidium iodide, and fractions of cells in different phases of cycle were quantified by flow cytometry. We found that neither did desialilation nor EGF, as well as erlotinib treatment, increase the number of damaged cells (subG0/G1 cell fraction), while erlotinib did significantly increase the number of G0/G1 cells and decrease S + G2/M cell fractions. In naive cells, EGF increased proliferating cell numbers by more than 40%, and this effect was blocked by erlotinib. In desialylated cells, however, proliferation was significantly decreased by about 29%, and EGF and erlotinib did not exert significant effects. We conclude that changes in alveolar epithelial cell membrane glycosylation may affect function of growth-promoting receptors and erlotinib effectiveness.

Keywords

A549 cells · Alveolar epithelial cells · Cell cycle · Epidermal growth factor · Erlotinib · Sialidase

1 Introduction

Erlotinib is classified as an epidermal growth factor (EGF) inhibitor, a cancer-targeted drug which is approved for treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC), after a failure of prior chemotherapy (Molina et al. 2008; Gridelli et al. 2007), and for advanced and metastatic pancreatic cancer (Moore et al. 2007). The mechanism of action of erlotinib is associated with epidermal growth factor receptor (EGFR) signaling pathways. Due to a reversible binding of erlotinib to the intracellular catalytic domain of EGFR, the receptor-

A. Rybak (✉), M. Zarzecki, E. Golabiewska, A. Niechoda, and A. Holownia
Department of Pharmacology, Medical University of Bialystok, Bialystok, Poland
e-mail: agatarybak22@gmail.com

dependent tyrosine kinase and receptor phosphorylation is blocked, and pro-proliferative effects of EGF are attenuated. Several publications indicate that cell sialylation impacts the proliferative properties of EGF (Britain et al. 2018; Park et al. 2012). In animal cells, sialylation is regulated by glycosyltransferases and glycosidases (Manhardt et al. 2017). Overexpression of neuraminidase-3 (NEU3), a member of glycosidase (neuraminidase/sialidase) family which hydrolyzes alpha-(2->3)-, alpha-(2->6)-, alpha-(2->8)-glycosidic linkages of terminal sialic acid residues in oligosaccharides, glycoproteins, and glycolipids, may activate extracellular-signal-regulated kinase (ERK) and v-akt murine thymoma viral oncogene (AKT)/protein kinase-B (PKB) pathways in respiratory epithelial cells (Forcella et al. 2017; Yun et al. 2008). Both kinases are involved in cell proliferation and apoptotic signaling. Modification of glycosylation of respiratory airway epithelia by NEU1 also activates EGFR, while overexpression of NEU 1 decreases EGF-mediated autophosphorylation of EGFR (Lillehoj et al. 2012).

Aberrant glycosylation is typical for cancer cells, and specific sialylation profiles (sialomes) are associated with malignant properties of cancer cells, including invasiveness and metastatic potential (Vajaria et al. 2016). There are four types of mammalian sialidases identified to date, NEU1–NEU4, which are expressed in different regions of a cell (Miyagi and Yamaguchi 2012). Neuraminidases catalyze the removal of sialic acid residues from glycoproteins and glycolipids. According to published data, NEU1 and NEU4 activities may be associated with antiproliferative effects, while NEU3 overexpression is related to cancer progression (Wada et al. 2007).

The aim of this study was to examine the effect of sialidase (alpha-neuraminidase) on EGF-EGFR interaction and on erlotinib efficacy in human A549 alveolar epithelial cells. We addressed the issue by quantifying changes in the cell cycle of both naïve and desialylated A549 cells, stimulated by EGF in the presence and absence of erlotinib.

2 Methods

2.1 Cell Culture

A549 cells (ATCC), adenocarcinoma-derived human alveolar epithelial cells, were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) solution at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.2 Cell Treatment

A549 cells were pretreated overnight with α-neuraminidase (100 U/ml from *Clostridium perfringens*; New England Biolabs, Ipswich, MA), and then naïve and desialylated cells were grown for 24 h in DMEM supplemented with EGF (200 ng/ml), erlotinib (100 μM), or both compounds combined.

2.3 Cell Growth and Cytotoxicity Assay

Cell proliferation and cytotoxicity were assessed using a stoichiometric DNA dye propidium iodide and cell cycle analysis in flow cytometry. Permeabilized A549 cells (0.1% NP-40) were stained for 30 min with propidium iodide (50 μg *per* ml) in trisaminomethane (TRIS) buffer (100 mM; pH 7.5), containing 0.1% potassium cyanide, 40 μg *per* ml of Type III-A RNAse, and 0.1% NaN_3. DNA profiles and cell cycle analysis were performed in the aligned FACSCanto II flow cytometer (BD Biosciences Systems; San Jose, CA) with a standard filter setup, equipped with an argon laser operating at 488 nm, having adjusted forward angle and side light scatter. Propidium iodide fluorescence was measured in 5000–10,000 cells, and DNA fluorescence histograms were analyzed by MultiCycle Software (Phoenix Flow Systems; San Diego, CA) and by flowing software v2.5 (Turku Center for Biotechnology; Turku, Finland). The cells were

quantified by their relative distribution in the hypodiploid or damaged (subG0/G1 zone of DNA fluorescence histograms), diploid (G0/G1 zone – pre-DNA synthesis/resting), S (DNA synthesis), and G2/M (post-DNA synthesis/mitosis) phases of the cell cycle. The percentage of cells in the subdiploid regions of histograms was considered an index of cytotoxicity, while S + G2/M cell numbers were quantified as proliferating fraction.

2.4 Statistical Analysis

Data were expressed as means ±SD of 4–6 assays. Statistical differences were evaluated with one-way or two-way ANOVA followed by Bonferroni post hoc test for selected pairs of data. A p-value <0.05 was defined statistical significance of differences. Statistical analysis was performed with a commercial Statistica 6.0 package (Statsoft; Cracow, Poland).

3 Results

Table 1 shows the effect of EGF, erlotinib, and both compounds combined on the cell cycle of A549 cells. Cells at different stages of growth were quantified as a fraction (%) of total cell number and were classified according to their DNA content and cell cycle phase, as shown in Fig. 1.

There was no significant toxicity in all experimental groups as evidenced by determination of subG0/G1 cell fractions. In naïve cells, EGF significantly increased the proliferating cell numbers and proliferation index by >40% ($p < 0.01$) and decreased the numbers of resting cells by 27% ($p < 0.01$) (Table 1 and Fig. 1). Erlotinib decreased cell proliferation, normalized resting cell numbers, and decreased the mitotic cell numbers by 17% ($p < 0.05$) and by 35% ($p < 0.01$), respectively.

In naïve cells, erlotinib normalized the pro-proliferative effects of EGF, without a significant effect on cell cycle arrest. However, when A549 cells were treated with neuraminidase, their proliferation was impeded, as evidenced by a 45% increase in resting G0/G1 cells ($p < 0.05$) and by a 29% decrease in fractions of proliferating S + G2/M cells ($p < 0.01$). Desialylated cells were neither stimulated by EGF nor inhibited by erlotinib.

4 Discussion

Activation of EGFR increases cell proliferation and angiogenesis (Lindsey and Langhans 2015). EGFR is activated by several peptide growth factors, and their binding induces receptor dimerization, autophosphorylation, and activation of downstream signaling pathways (Mitsudomi and Yatabe 2010; Schlessinger 2000). EGFR-dependent kinases are widely studied targets for anticancer drugs, since EGFR tyrosine kinase inhibitors, such as erlotinib, gefetinib, and afatinib, have proven effectiveness in some types of cancer. EGFR is a heavily glycosylated protein, and its sialylation suppresses receptor activation and drug response (Britain et al. 2018). Desialylation decreases sensitivity of lung cancer cells to gefitinib, and this effect is reversed, at least in part, by sialylation (Yen et al. 2015).

The aim of the present study was to examine the effect of sialidase on EGF-EGFR interaction and on the efficacy of erlotinib in the human A549 alveolar epithelial cell line. We addressed the issue by quantifying changes in the cell cycle of naïve and desialylated cells, expressing wild-type EGFR, stimulated by EGF in the presence and absence of EGF. Our results indicate that erlotinib is not toxic to A549 cells, at least in acute exposure, even though the drug concentration we used was significantly higher than EC50. Erlotinib produced cell cycle arrest at G0/G1, which has already been described in a similar cellular model (Shan et al. 2016). According to the published data, erlotinib can increase oxidative stress and induce apoptosis (Orcutt et al. 2011). In the present experiments, erlotinib abolished the EGF-induced proliferation of A549 cells, but it was only modestly effective in unstimulated cells. This observation seems

Table 1 Analysis of cell proliferation and cytotoxicity in naïve and sialidase-treated (100 U/ml) A549 cells grown for 24 h with epidermal growth factor (EGF; 200 ng/ml), erlotinib (100 μM), or with both compounds combined. Cells were stained with propidium iodide and quantified in flow cytometry by their relative distribution in the hypodiploid (subGO/G1), diploid (G0/G1), and S + G2/M phases of the cell cycle

	Control cells			EGF			Erlotinib			EGF + erlotinib		
	SubG0/G1	G0/G1	S + G2/M	SubG0/G1	G0/G1	S + G2/M	SubG0/G1	G0/G1	S + G2/M	SubG0/G1	G0/G1	S + G2/M
Control	7 ± 5	59 ± 11	34 ± 11	9 ± 5	43 ± 12**	48 ± 12**	9 ± 4	69 ± 9*$^{\#\#}$	22 ± 8**$^{\#\#}$	11 ± 4	58 ± 10$^{\#\#}$	31 ± 12$^{\#\#}$
Sialidase	9 ± 4	67 ± 13**^	24 ± 8**	11 ± 6	61 ± 11^^	28 ± 10$^{\#\#}$^^	11 ± 5	68 ± 12	21 ± 8$^{\#}$	12 ± 5	62 ± 13	26 ± 11$^{\#}$

Data are means ± SD; *p < 0.05 and **p < 0.01 vs. control (naive or sialidase pretreated); #p < 0.05 and ##p < 0.01 vs. EGF-treated cells; ^p < 0.05 and ^^p < 0.01 vs. corresponding control

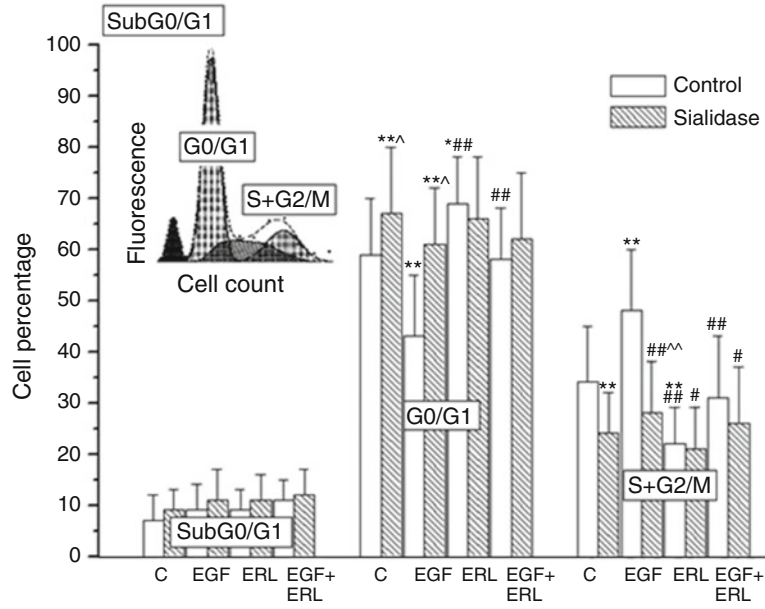

Fig. 1 Cell proliferation and cytotoxicity in naïve and sialidase-treated (100 U/ml) A549 cells grown for 24 h with epidermal growth factor (EGF; 200 ng/ml), erlotinib (ERL; 100 μM), or with both compounds combined. Cells were stained with propidium iodide and quantified in flow cytometry by their relative distribution in the hypodiploid (sub-G0/G1), diploid (G0/G1), and S + G2/M phases of the cell cycle as shown in the fluorescence histogram. Data are as means ± SD; *p < 0.05 and **p < 0.01 vs. control (naive or sialidase pretreated); #p < 0.05 and ##p < 0.01 vs. EGF-treated cells; and ^p < 0.05 and ^^p < 0.01 vs. corresponding control

clinically relevant, considering a high pro-proliferative potential of EGFR agonists. In fact, NEU3 has been proposed as a diagnostic marker in non-small cell lung cancer (NSCLC) that helps distinguish between gefitinib-responsive and nonresponsive patients without EGFR mutations (Forcella et al. 2017). On the other hand, when our cells were treated with neuraminidase, their proliferation was impeded, as evidenced by an increased fraction of resting cells and a decreased fraction of proliferating cells. Several studies have shown that glycans can participate in the regulation of EGFR function. Both sialylation and fucosylation of EGFR suppress receptor dimerization, autophosphorylation, and invasiveness of cancer cells (Takahashi et al. 2016; Liu et al. 2011). In the present experimental model, the effect of extracellular desialylation of the cell membrane, and possibly also of EGFR, was examined to understand how sialidase could influence the receptor response to an agonist in wild-type EGFR cells. Our results demonstrate that extracellular desialylation could suppress the EGFR response, possibly by affecting EGF binding. It has been earlier shown that desialylated cells with mutated EGFR are resistant to gefitinib (Sharma et al. 2007). Both erlotinib and gefitinib are reversible kinase inhibitors, but they have quite distinct chemical, pharmacokinetic, and clinical properties. The most striking difference is that gefitinib is effective mostly in EGFR-mutant patients, while erlotinib also is in EGFR-wild-type patients (Wu et al. 2012). EGFR sialylation may suppress its dimerization and phosphorylation (Yen et al. 2015). However, in a model with mutated EGFR, both sialidase and sialyltransferase inhibitors show increased receptor phosphorylation, while in parallel experiments with gefitinib-resistant lung cancer cells, drug sensitivity is significantly reduced by desialylation and enhanced by sialylation (Britain et al. 2018). The present findings indicate that desialylated A549 cells were neither stimulated by EGF nor inhibited by erlotinib. Since in our experiments erlotinib was able to obliterate the effect of EGF, with only a small influence on unstimulated cells, it is not surprising that clinical

observations on patients with EGFR mutations and with constitutive receptor activation show a high initial effectiveness of erlotinib (Mitsudomi and Yatabe 2010; Sharma and Settleman 2009).

In conclusion, changes in alveolar epithelial cell membrane glycosylation may affect the function of growth-promoting receptors and erlotinib efficacy. Further studies on EGFR sialylation patterns should provide a better understanding of receptor homodimerization- and heterodimerization-related processes, especially in the context of TKI resistance and cancer progression.

Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.
This article does not contain any studies with human participants or animals performed by any of the authors.

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Systemic Sclerosis and Serum Content of Transforming Growth Factor

Dominik Majewski, Katarzyna A. Majewska, Barbara Kuznar-Kaminska, Marta Runowska, Tomasz Piorunek, Halina Batura-Gabryel, and Mariusz Puszczewicz

Abstract

Systemic sclerosis is a connective tissue disease characterized by tissue fibrosis leading to interstitial lung disease. Transforming growth factor-β (TGF-β) has been of interest as a potential diagnostic marker and also as a drug target in systemic sclerosis. The aim of this study was to assess the serum content of TGF-β1 in patients with systemic sclerosis and to assess its potential role in tissue fibrosis. The study included 30 patients, 5 men and 25 women, of the mean age of 46.9 ± 12.8 years, diagnosed with systemic sclerosis. The control group consisted of 19 women of the mean age of 28.4 ± 7.8 years, diagnosed with primary Raynaud's disease. TGF-β1 serum levels were measured, chest imaging examinations were performed, and fibrotic tissue changes were assessed using the modified Rodnan Skin Score. We found that the mean serum TGF-β1 content in patients with systemic sclerosis was 598.7 ± 242.6 pg/mL, whereas it was 568.4 ± 322.2 pg/mL in the control group ($p = 0.378$). We also failed to substantiate any significant relationship between TGF-β1 serum levels and the severity of pulmonary and skin fibrosis in systemic sclerosis. In conclusion, systemic sclerosis does not seem a disease that would be accompanied by a specific enhancement of serum TGF-β1. Thus, this cytokine is rather unlikely to play an essential role in the development and course of the disease, nor can it be considered diagnostic or prognostic marker.

Keywords

Connective tissue disease · Cytokines · Fibroblasts · Pulmonary hypertension · Rheumatology · Scleroderma

D. Majewski (✉), M. Runowska, and M. Puszczewicz
Department of Rheumatology and Internal Diseases, Poznan University of Medical Sciences, Poznan, Poland
e-mail: dmajes@poczta.onet.pl

K. A. Majewska
Department of Clinical Auxology and Pediatric Nursing, Poznan University of Medical Sciences, Poznan, Poland

B. Kuznar-Kaminska, T. Piorunek, and H. Batura-Gabryel
Department of Pulmonology, Allergology and Respiratory Oncology, Poznan University of Medical Sciences, Poznan, Poland

1 Introduction

Systemic sclerosis is a connective tissue disease characterized by tissue fibrosis leading to multiple organ impairment. The disease most often affects the skin, subcutaneous tissue, and internal organs, especially the lungs, kidneys, gastrointestinal tract, and heart. The pathogenesis of the disease remains unclear, but disorders of the immune

system leading to the production of autoantibodies against topoisomerase I (anti-Scl-70), against centromeres, and other factors are of significant importance. The occurrence of morphological changes and vascular dysfunction also play a role in the perpetuation of the disease. As a result of these processes, fibroblasts are activated, and excessive amounts of collagen are produced. Collagen accumulates and progressive fibrosis occurs, destructing tissue. One of the cytokines involved in systemic sclerosis is the transforming growth factor-β (TGF-β) and its isoform TGF-β1 that is related to the activity of connective tissue cells (Lafyatis 2014; Collier 2002; Klippel and Dieppe 1998). TGF-β has its receptors in virtually all eukaryotic human cells. It is believed that in systemic sclerosis, TGF-β stimulates fibroblasts to produce extracellular matrix components and inhibits enzymes that degrade them. An often reported organ complication of systemic sclerosis is fibrosis of lung tissue, known as interstitial lung disease, which may result in the development of secondary pulmonary hypertension (Piorunek et al. 2013). One piece of evidence of the TGF-β role in the pathogenesis of systemic sclerosis is the fact that in animal models of diseases with fibrosis, administration of antibodies against TGF-β prevents excessive production of connective tissue and consequent destruction of organs (Leask 2006; Verrecchia et al. 2006; Border and Noble 1994; Border and Ruoslahti 1992). Recently, TGF-β has been of interest as a diagnostic and prognostic marker and also as a drug target in systemic sclerosis and some other diseases (Wermuth and Jimenez 2018; Du et al. 2017; Jakubowska et al. 2015; Piotrowski et al. 2015). Therefore, the present study seeks to define the content of serum TGF-β in patients with systemic sclerosis and to assess its potential value in the early diagnosis of systemic sclerosis-related tissue fibrosis.

2 Methods

The study consisted of 30 patients (F/M; 25/5) of the mean age of 46.9 ± 12.8 years, with the diagnosis of systemic sclerosis based on the criteria of the American Rheumatism Association (Collier 2002). Duration of systemic sclerosis ranged from 1 to 20 years (mean 8.1 ± 5.3 years). The control group consisted of 19 women, aged from 20 to 48 (mean 28.4 ± 7.8) years whose diagnosis was the primary Raynaud disease.

The presence of interstitial lung disease was established by chest X-rays in the posterior-anterior projection and by high-resolution computed tomography. Pulmonary hypertension was diagnosed on the basis of clinical signs such as progressive dyspnea, fatigue, and chest pain resulting in limitation of patients' exercise tolerance and on the estimated pulmonary artery pressure exceeding 35 mmHg in Doppler echocardiography (Collier 2002). The following score of organ changes was used in this study:

0 – no evidence of interstitial lung disease and pulmonary hypertension
1 – interstitial changes involving basal lung fields
2 – interstitial changes involving middle and upper lung fields
3 – interstitial changes involving middle and upper lung fields and pulmonary hypertension.

To evaluate the skin involvement in patients with systemic sclerosis, a modified Rodnan Skin Score (mRSS) was used. The hardening of skin areas was assessed on a 4-point scale: 0, normal; 1, slight hardening; 2, moderate hardening; and 3, significant skin hardening. This assessment involves both the degree of skin hardening and the extent of skin involvement. The total mRSS score ranges from 0 to 51 points (Denton and Black 2004; Akesson et al. 2003; Clements 2000).

The TGF-β1 content was evaluated with a commercial Quantikine human TGF-β1 kit (R&D Systems, Minneapolis, MN), based on the ELISA method. The test enables the quantitative assessment of activated TGF-β1. The minimum detectable concentration of TGF-β1 was in a range of 1.7–15.4 pg/mL. However, the manufacturer of the test kit does not specify the range of a normal serum level of the cytokine since it is not defined. Thus, TGF-β1 level found in this study was compared with that reported in

previous studies performed in patients suffering from systemic sclerosis and in healthy persons.

Data were presented as means ±SD. Differences between the two groups were evaluated with a two-tailed *t*-test. A p-value <0.05 defined statistically significant differences. The evaluation was conducted using a commercial STATISTICA package (StatSoft; Tulsa, OK).

3 Results

The mean serum TGF-β1 content in patients with systemic sclerosis was 598.7 ± 242.6 pg/mL, whereas in the control group, consisting of patients with primary Raynaud's disease, it was 568.4 ± 322.2 pg/mL ($p = 0.38$) (Fig. 1). The maximum levels of 1250.0 pg/mL and 1310.0 pg/mL and minimum levels of 200.0 pg/mL and 270.0 pg/mL, respectively, were not significantly different either.

Skin hardening was observed in all of the systemic sclerosis patients, with the mean mRSS score of 9.7 ± 9.2 and the min-max range of 1–34 points. A lung involvement with characteristic interstitial fibrotic changes was noticed in 19 (63.4%) out of the 30 systemic sclerosis patients. This involvement was confined to basal lung segments in 12 (40.0%) and to the middle and upper lung fields in 5 (16.7%) patients. In 2 (6.7%), patients, the features of interstitial lung disease were accompanied by pulmonary hypertension.

A relation between the severity of skin involvement, evaluated by mRSS scale, and the serum TGF-β1 content in systemic sclerosis patients turned out insignificant ($p = 0.476$; $r = 0.14$). Likewise, TGF-β1 content failed to relate to the appearance of lung involvement in the patients ($r = 0.32$).

4 Discussion

The findings of this study failed to confirm the existence of any relationship between the serum TGF-β1 content and the appearance and severity of pulmonary and skin fibrosis in patients with systemic sclerosis. We confronted these findings with the previous literature data on the subject of TGF-β1 in systemic sclerosis. Scala et al. (2004) did not observe a significant difference in the content of total TGF-β1 between patients with a limited as well as generalized form of systemic sclerosis and control subjects, with the mean TGF-β1 of 3499 ± 2357 pg/mL, 3552 ± 2357 pg/mL, and 3542 ± 4410 pg/mL, respectively. Snowden et al. (1994) detected TGF-β1 in the plasma of 6 out of the 39 patients

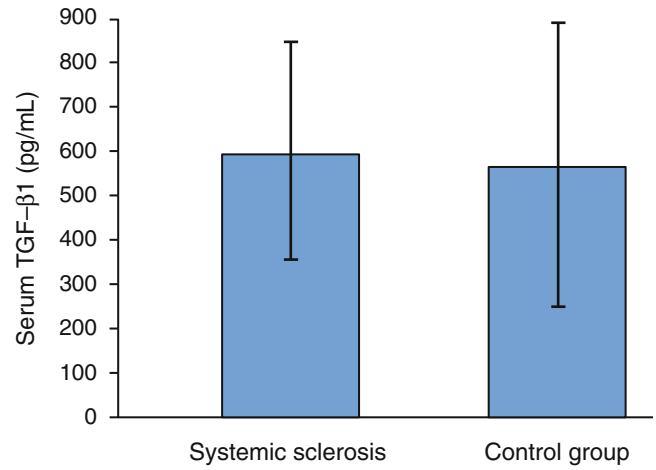

Fig. 1 Serum TGF-β1 content is systemic sclerosis and control patients. Data are means ±SD

with systemic sclerosis, employing a test having the minimum detectable level of 100 pg/mL, i.e., being more than sixfold less sensitive for TGF-β1 detection than the test used in the current study. This level of detection was not reached in serum samples from any of the 60 healthy subjects and 9 patients with Raynaud's disease, implying a tendency for a higher TGF-β1 content in some patients with systemic sclerosis. In contradistinction, Dziadzio et al. (2005) reported a reduced content of the active form of TGF-β1 in patients with scleroderma, in particular in the generalized disease, compared to the control subjects, with the median TGF-β of 520 pg/mL and 1,230 pg/mL, respectively. These authors, however, failed to notice any significant reduction in the total content of TGF-β1 in scleroderma. The opposite results were reported by Dantas et al. (2016) who show an increase in active TGF-β1 and its association with clinical manifestations of scleroderma. Therefore, the issue of the content and role of TGF-β1 in the course of systemic sclerosis is highly contentious, and the data are discrepant. The issue is further compounded by as yet undetermined normal level of serum TGF-β1 in the general population. In addition, discrepancy in TGF-β1 values may stem from the assessment of active versus total TGF-β1 or using different not standardized commercial kits. In the current study, the active form of TGF-β1 showed a tendency for elevation in systemic sclerosis patients, which seems somehow in line with the findings of Dziadzio et al. (2005). Scala et al. (2004), on the other side, who showed no changes in serum TGF-β1 in various types of scleroderma, investigated the total TGF-β1 content. The accuracy of TGF-β1 measurement may be highly affected by the fact that only a part of this protein, having a mass of 25 kDa, is in the active form that has a very short half-life in bodily fluids. Wakefield et al. (1990) reported the half-life of active TGF-β1 as short as 2–3 min, compared with more than a 100 min for the latent TGF-β1 form. The existence of this dual form of TGF-β1 introduces a nuisance in the understanding of TGF-β1 bioactivity, which can hardly be resolved at the current state of knowledge. In addition, in the pathogenesis of systemic sclerosis, not so much the serum content as the ratio of active to latent TGF-β1 form could be a key factor. Other theories raise the possibility of overexpression of type I fibroblast receptors (Leask 2006; Border and Noble 1994) or disruption of the intracellular transduction cascade of TGF-β1 by a defective Smad protein as the mechanisms by which TGF-β affects tissue fibrosis (Verrecchia et al. 2006).

Of note, the control group of the current study consisted of patients with primary Raynaud's disease, which may enhance the predilection for or be a presage of systemic sclerosis (Cutolo et al. 2017). Nonetheless, we found no appreciable difference in the serum content of active TGF-β1 between patients with systemic sclerosis and Raynaud's disease, which strengthens the impression that this form of TGF-β1 may not be at play in shaping detrimental molecular and cellular changes underlying fibrosis in systemic sclerosis. To this end, our findings are in line with those of Snowden et al. (1994) who investigated the serum content of TGF-β1 in nine patients with Raynaud's disease, who were part of the control group, and found the undetectable level of it. In addition, we also failed to demonstrate any association between the serum content of TGF-β1 and fibrotic changes in the skin or the lungs, which is generally in line with data from previous studies (Dziadzio et al. 2005; Snowden et al. 1994). In conclusion, we believe that despite a biological plausibility of the stimulating role of TGF-β1 in the development of skin and other tissues' fibrotic changes in the course of systemic sclerosis, we found in this study no supportive evidence for such an action. A lack of changes in serum TGF-β1 also makes it unlikely that this cytokine could be considered a diagnostic marker or a marker of severity of skin or lungs involvement in systemic sclerosis. A multifarious and complex bioactivity of TGF-β1 remains contentious and is open to continuing exploration in other study designs.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national

research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Bioethics Committee of the Karol Marcinkowski University of Medical Sciences in Poznań, Poland.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Protein-Bound Solute Clearance During Hemodialysis

Małgorzata Gomółka, Longin Niemczyk, Katarzyna Szamotulska, Aleksandra Wyczałkowska-Tomasik, Aleksandra Rymarz, Jerzy Smoszna, Mariusz Jasik, Leszek Pączek, and Stanisław Niemczyk

Abstract

Indoxyl sulfate (IS) and p-cresol sulfate (p-CS) are protein-bound solutes that accumulate in the blood serum in chronic kidney disease and have a detrimental effect on the kidney and other organs' function. This study seeks to define the effectiveness of IS and p-CS clearance after single dialysis sessions and after 8-week-long cycles of hemodialysis using the following different dialysis modalities in succession: low-flux hemodialysis (lfHD), high-flux hemodialysis (hfHD), and post-dilution hemodiafiltration (HDF). We also investigated to what extent IS and p-CS serum content would associate with some other biochemical indices in patients with chronic kidney diseases. The study included 21 uremic patients. We found that a single session of each modality effectively decreased the content of both IS and p-CS, with the predominance of p-CS decrease. There were no appreciable differences depending on the modality of hemodialysis chosen. However, the leaching effect tended to wear off with the weeks' long dialysis cycles. We further found that a greater inflammation-prone level of hsCRP evoked by dialysis led to a greater removal of solutes, and thus their decrease in the serum, during a single dialysis session. Reversely, a greater protein level might result in a greater solute binding and a decrease in removal. We conclude that there are no major differences in the serum clearance of IS and p-CS depending on the dialysis modality. These protein-bound toxins are significantly cleared from the serum already during the first dialysis session, but their level tends to revert during weeks' long dialysis sessions.

Keywords

Blood toxin clearance · Cresol sulfate · Hemodiafiltration · Hemodialysis · Indoxyl sulfate · Protein-bound solutes

M. Gomółka, A. Rymarz, J. Smoszna, and S. Niemczyk
Department of Internal Medicine, Nephrology and Dialysis, Military Institute of Medicine, Warsaw, Poland

L. Niemczyk (✉)
Department of Nephrology, Dialysis and Internal Medicine, Warsaw Medical University, Warsaw, Poland
e-mail: lniemczyk@wum.edu.pl

K. Szamotulska
Department of Epidemiology and Biostatistics, Institute of Mother and Child in Warsaw, Warsaw, Poland

A. Wyczałkowska-Tomasik and L. Pączek
Department of Immunology, Transplantology and Internal Medicine, Warsaw Medical University, Warsaw, Poland

M. Jasik
Department of Obstetrics and Gynecology, Warsaw Medical University, Warsaw, Poland

1 Introduction

Indoxyl sulfate (IS) and p-cresol sulfate (p-CS) are protein-bound solutes which are not routinely assessed in the clinical setting. Adverse effects of protein-bound solutes have been reported both in vitro and in vivo. These effects, in the main, consist of glomerulosclerosis, interstitial tissue fibrosis, and extracellular matrix deposits in kidneys (Shimizu et al. 2011). Protein-bound solutes activate oxygen free radical formation, transforming growth factor-β1 (TGF-β1), and nuclear factor kappa B (NF-κB) expressions, and they decrease Klotho protein expression (Lekawanvijit 2015; Itoh et al. 2012). IS activates oxidative stress by increasing the production of NAD(P)H oxidase and reducing that of glutathione. IS and p-CS induce epithelial-to-mesenchymal transition, a biological process in which cells with the epithelial phenotype transform into cells with the mesenchymal phenotype. Under the influence of protein-bound solutes, the intrarenal renin-angiotensin-aldosterone system is activated (Sun et al. 2012; Natsuizaka et al. 2010). In the kidney, epithelial-to-mesenchymal transition leads to fibrosis in both glomeruli and interstitial tissue (Lekawanvijit 2015). By increasing the activity of aryl hydrocarbon receptor, IS damages podocytes, the cells that are crucial for proper functioning of glomeruli (Ichii et al. 2014). The uremic toxins IS and p-CS contribute to the accelerated development of atherosclerosis in patients with chronic kidney disease due to facilitation of calcium-phosphate metabolism disorders and anemia (Foley et al. 1998). Standard hemodialysis scarcely removes protein-bound solutes, and the removal applies only to their free fraction (Dou et al. 2007; Sun et al. 2017).

The unfavorable effects of protein-bound solutes on the organism are thought to contribute to increased morbidity and mortality in patients with chronic kidney disease, although some studies have failed to report the presence of serious adverse cardiovascular outcomes (Shafi et al. 2017). Therefore, the present study seeks to define the effectiveness of IS and p-CS removal after a single dialysis session and after an 8-week-long dialysis course, using successively the following hemodialysis modalities: low-flux hemodialysis (lfHD), high-flux hemodialysis (hfHD), and post-dilution hemodiafiltration (HDF). In addition, we investigated whether enhanced IS and p-CS serum content would associate with some other biochemical indices in patients with chronic kidney diseases.

2 Methods

2.1 Patients

Twenty-one patients (8F, 13 M) were enrolled into the study, including eight individuals (3F, 5 M) with diabetic nephropathy (38%). The patients were in a stable clinical condition on renal replacement therapy for a minimum of 3 months (three treatments per week), with a maintenance dialysis standard urea $Kt/V > 1.2$, where K is urea clearance, t is dialysis time, and V is urea distribution volume. The consent for participation in the study was withdrawn by one patient, and one other patient died due to infective endocarditis and sepsis. A single-treatment run in each dialysis modality was completed by 19 patients. However, only 18 patients were taken into account for the final analysis of toxin clearance after all three modalities of therapy, each taking 8 weeks (see the protocol below), since the ending measurement of p-CS was missing in one patient. Patients with clinically overt inflammation, autoimmune diseases, neoplastic disease, malnutrition (BMI < 19.0 kg/m^2), thyroid disorders, severe liver damage, blood coagulation disorders, remaining on hormonal therapy or oral anticoagulant treatment, were excluded from the study.

2.2 Study Protocol

The study lasted for 24 weeks and consisted of three dialysis modalities: I, lfHD (Braun LOPS); II, hfHD (Braun HIPS); and III, HDF with the mean volume of post-dilution substitute of 14.1 ± 1.5 L/treatment. Each modality consisted

of three sessions *per* week for 8 weeks. Blood for the evaluation of IS and p-CS content in the serum was collected before and after the first single session of each dialysis modality and at the end of the third dialysis modality ending the study protocol, i.e., after 24 weeks. In this paradigm, the serum levels of IS and p-CS drawn at the end of lfHD corresponded to the baseline levels of both toxin before the next hfHD modality. Likewise, content of blood drawn at the end of hfHD corresponded to the baseline blood content before HDF. Altogether, blood was drawn seven times in each patient (Fig. 1). The effects of each dialysis modality on the IS and p-CS content were evaluated relative to the corresponding baseline level of these toxins.

Dalteparinum natricum was used for anticoagulation. The content of IS and p-CS was evaluated with high-performance liquid chromatography (HPLC) (Merck; Darmstadt, Germany). The content of albumins was determined with a colorimetric method using bromocresol green (Olympus AU 680 analyzer; Beckman Coulter, Brea, CA). Prealbumins and high specificity C-reactive protein (hsCRP) were determined with a nephelometric method (BN II analyzer; Siemens, Munich, Germany) and insulin with a chemiluminescence method (Liaison analyzer; DiaSorin, Saluggia, Italy). The contents of tumor necrosis factor (TNF-α) and interleukins (IL)-1β and IL-6 were measured with Luminex – Human HS Cytokine Panel A (R&D Systems; Minneapolis, MN) – and those of visfatin and leptin with ELISA method (DRG MedTek; Warsaw, Poland).

2.3 Statistical Evaluation

Data were presented as means ±SD. The Wilcoxon test and the Friedman test for small samples were used for the statistical comparison of differences in the content of biochemical indices measured. The latter test was used to compare changes over the three treatment runs of each dialysis modality over time. The generalized estimating equations (GEE) of a generalized linear model were employed to estimate the relationships between multiple changes in IS and p-CS serum content and multiple changes in the biochemical indices investigated during various modes of dialysis. A p-value <0.05 defined statistically significant changes. A commercial SPSS v24 statistical package (IBM Corp, Armonk, NY) and R3.3.4 package were used.

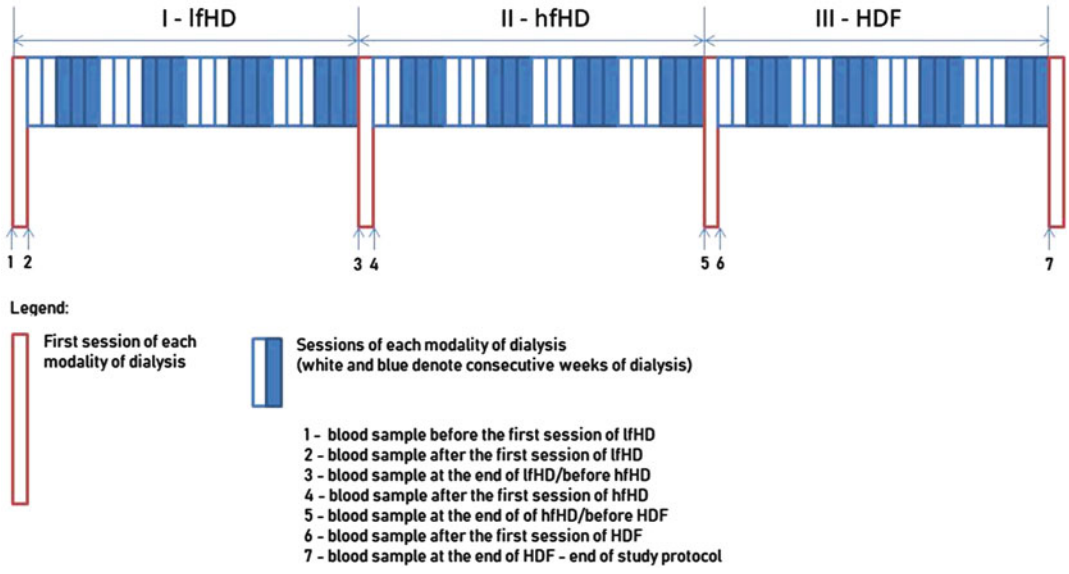

Fig. 1 Study paradigm. Modalities of dialysis: I, low-flux hemodialysis (lfHD), high-flux hemodialysis (hfHD), and post-dilution hemodiafiltration (HDF)

3 Results

Out of the 19 patients (7 K/11 M), in 6 individuals (31.6%) chronic kidney disease was due to diabetic nephropathy. At the study onset, patients were 54.5 ± 14.5 (29.7–71.8) years old, had the mean body mass index of BMI of 28.6 (19.3–43.0) kg/m^2, and the mean 24-h diuresis of 737 mL. Two patients had anuria. The mean period of renal replacement therapy in the hemodialysis program was 20.0 ± 14.4 (3.7–61.2) months. Single treatment lasted for 236.8 ± 24.3 (180–270) min, and it was not modified during the study. The volume of the substitute was 14.1 ± 1.5 (10.8–16.2) L/treatment.

Table 1 shows clinical characteristics of the study group. Albumin and prealbumin contents were stable throughout the observation period, as was TNF-α. The serum levels of IL-1β and hsCRP tended to increase after each 8-week-long runs of consecutive dialysis modalities, as opposed to IL-6 that decreased.

Table 2 presents changes in the serum content of IS and p-CS after the first single session of each dialysis modality. A significant decrease in the mean serum content of IS and p-CS during each dialysis modality was noticed ($p < 0.001$). However, there were no differences in the absolute (Δ) and relative (Δ%) differences in the decreases between individual modalities of dialysis. When the full-length 8-week-long cycles of each dialysis modality were considered, no significant pre-post cycle differences were noticed in either IS or p-CS content in each modality or among the three modalities of dialysis (Table 3).

When studying the relation between changes in IS or p-CS and biochemical indices, triggered by 8-week-long lfHD, hFHD, and HDF dialysis, we noticed a positive association between p-CS and both albumin ($p < 0.001$) and prealbumin ($p = 0.001$) and an adverse association between p-CS and hsCRP content ($p = 0.002$). The associations between changes in IS and both albumin and hsCRP were similar in direction to those in case of p-CS, reaching just borderline significance, and the only significant association was between IS and the proinflammatory IL-1β (Table 4).

4 Discussion

Molecular weight of a majority of protein-bound solutes is <500 Da. However, IS and p-CS are strongly bound to proteins, mainly to albumins, which means that current dialysis techniques are not sufficiently effective in their removal. Clearance of protein-bound solutes depends on the concentration of free fractions, on the changing balance between free fraction and albumin-related proportion in the course of hemodialysis, and on the quantity of solutes present in the extravascular space. During hemodialysis, serum solutes are removed in about 30% while urea in 60% (Ito and Yoshida 2014; Tattersal et al. 2013; Meert et al. 2010; Foley et al. 1998). The findings of the present study, in general, confirm the observations above outlined. Furthermore, we found that reductions in IS and p-CS content before and after a single dialysis session were significant in all stages of the study. However, this difference bleared out during the 8-week-long cycles with each of the three modalities of dialysis (lfHD, hfHD, and HDF). We also found a tendency for greater p-CS removal during HDF, compared to lfHD and hfHD. Sirich et al. (2017) have reported that frequency and duration of HDF have no effect on the removal of protein-bound solutes. At the same time, a greater removal is associated with a faster rebound in the solute content, so that they may not appreciably change before the next treatment. Therefore, it is advisable to inhibit the formation of these toxic solutes in the gastrointestinal tract, which is conducive to the effectiveness of dialytic treatment (Nazzal et al. 2017; Camacho et al. 2016).

In this study we found that the more protein there is, the worse p-CS removal, to a lesser extent that applies to IS. This suggests a different strength of binding of both toxins to proteins, in favor of p-CS, in patients with uremia, although a previous in vitro study has not differentiated the binding strength of the two solutes (Deltombe et al. 2017). During HDF, only is a free fraction of a solute removed from the circulation. Infections may lead to an increase in free fractions of serum solutes, which is greater for p-CS

Table 1 Clinical characteristics of patients. The indices were measured in the serum before each 8-week-long run of dialysis modality and then after the protocol end, i.e., after 24 weeks of dialysis

Biochemical indices	*n*	Before/ baseline	After lfHD/before hfHD	After hfHD/before HDF	After HDF/end of study
Albumins (g/dL)	19	3.9 ± 0.2	3.8 ± 0.3	3.7 ± 0.2	3.7 ± 0.4
		(3.5–4.2)	(3.3–4.5)	(3.4–4.1)	(2.4–4.3)
Prealbumins (mg/dL)	19	31.9 ± 6.8	32.4 ± 8.1	30.2 ± 5.8	31.5 ± 8.5
		(22.0–47.0)	(24.0–56.0)	(21.0–45.0)	(11.0–51.0)
hsCRP (mg/dL)	19	0.8 ± 0.8	0.9 ± 1.0	1.1 ± 1.9	2.1 ± 5.3
		(0.0–3.5)	(0.0–4.4)	(0.0–8.4)	(0.0–23.6)
IL-1β (pg/mL)	15	2.33 ± 3.6	2.4 ± 4.2	2.9 ± 4.2	5.3 ± 17.7
		(0.4–13.9)	(0.4–15.4)	(0.4–15.3)	(0.4–69.2)
IL-6 (pg/mL)	15	7.4 ± 9.1	5.9 ± 4.0	5.0 ± 4.1	5.4 ± 2.7
		(1.4–38.2)	(1.6–16.4)	(1.6–13.0)	(2.2–13.4)
TNF-α (pg/mL)	15	30.9 ± 8.6	30.6 ± 8.4	30.7 ± 6.9	28.9 ± 6.6
		(19.3–51.6)	(20.6–46.4)	(18.7–45.9)	(18.1–40.5)
Insulin (μIU/mL)	19	18.6 ± 22.3	21.4 ± 18.2	19.1 ± 16.9	19.4 ± 19.2
		(3.6–105.6)	(3.7–71.1)	(6.1–72.0)	(3.1–84.7)
Leptin (ng/mL)	19	35.52 ± 38.0	36.2 ± 38.9	36.4 ± 42.2	38.7 ± 42.0
		(0.0–133.2)	(0.0–125.9)	(0.1–147.6)	(0.0–144.6)
Visfatin (ng/mL)	19	1.0 ± 0.7	1.0 ± 0.7	1.1 ± 0.7	1.0 ± 0.7
		(0.3–2.4)	(0.2–2.7)	(0.2–2.8)	(0.2–2.7)

Data are means ±SD (minimum-maximum range)
lfHD low-flux hemodialysis, *hfHD* high-flux hemodialysis, *HDF* post-dilution hemodiafiltration, *hsCRP* high sensitivity C-reactive protein, *IL* interleukin, *TNF-α* tumor necrosis factor alpha

Table 2 Changes in serum indoxyl sulfate (IS) and p-cresol sulfate (p-CS) content after the first single session of each of the following: low-flux hemodialysis (lfHD), high-flux hemodialysis (hfHD), and post-dilution hemodiafiltration (HDF)

Biochemical indices	Dialysis modality	Pre-single session	Post single session	Δ Pre-post	p^{Δ}	Δ% Pre-post	$p^{\Delta\%}$
IS (mg/L)	lfHD	25.1 ± 11.2	19.0 ± 8.6	−6.1 ± 4.5	<0.001	−22.9 ± 17.3	0.398
	hfHD	23.9 ± 12.0	17.3 ± 8.6	−6.6 ± 4.8	<0.001	−25.5 ± 15.7	
	HDF	24.7 ± 10.3	16.2 ± 7.9	−8.5 ± 4.5	<0.001	−34.1 ± 12.4	
p-CS (mg/L)	lfHD	45.3 ± 19.1	36.8 ± 14.5	−8.5 ± 7.8	<0.001	−17.6 ± 13.2	0.144
	hfHD	41.9 ± 21.5	33.4 ± 15.5	−8.6 ± 10.4	<0.001	−18.6 ± 13.3	
	HDF	38.5 ± 22.0	28.7 ± 19.2	−9.7 ± 6.1	<0.001	−14.5 ± 54.6	

Data are means ±SD
p^{Δ} denotes significance of differences in the protein-bound IS and p-CS toxins investigated pre-post single session of a given dialysis modality, $p^{\Delta\%}$ denotes significance of relative differences in magnitude of either toxin reduction among the three modalities of dialysis

(Banerjee et al. 2017). The present findings seemed to confirm that possibility, pointing to an inverse relation between the level of hsCRP and that of both toxins, i.e., their greater clearance from the blood during dialysis due likely to an increase in their free fractions, after a single dialysis session. This effect, however, evanesced with time of the 8-week-long cycles of dialysis.

During lfHD, molecules <500 Da soluble in water are removed by diffusion. hfHD combines diffusion with convection, which enables removal of substances of up to 40 kDa. However, the estimated amount of convective transport during hfHD is insignificant (<10 L/treatment). In HDF, which also combines diffusion with convection, convective transport is much higher and

Table 3 Changes in serum indoxyl sulfate (IS) and p-cresol sulfate (p-CS) content before and after 8 weeks' cycles of low-flux hemodialysis (lfHD), high-flux hemodialysis (hfHD), and post-dilution hemodiafiltration (HDF)

Biochemical indices	Dialysis modality	Pre 8 weeks' dialysis cycles	Post 8 weeks' dialysis cycles	Δ Pre-post	p^{Δ}	Δ% Pre-post	$p^{\Delta\%}$
IS (mg/L)	lfHD	25.9 ± 11.5	25.0 ± 12.6	−1.0 ± 5.1	0.629	−5.1 ± 20.7	0.986
	hfHD	25.0 ± 12.6	25.9 ± 11.2	0.9 ± 4.7	0.260	10.3 ± 23.9	
	HDF	25.9 ± 11.2	26.7 ± 15.8	0.9 ± 8.7	0.768	−0.2 ± 33.8	
p-CS (mg/L)	lfHD	45.4 ± 18.6	42.6 ± 21.2	−2.8 ± 16.7	0.368	−0.5 ± 49.8	0.763
	hfHD	42.6 ± 21.1	38.1 ± 21.5	−4.6 ± 21.6	0.791	1.1 ± 50.6	
	HDF	38.1 ± 21.5	33.9 ± 25.1	−4.1 ± 18.6	0.308	137.9 ± 647.0	

Data are means ±SD

p^{Δ} denotes significance of differences in the protein-bound IS and p-CS toxins investigated pre-post 8 weeks' cycles of a given dialysis modality

$p^{\Delta\%}$ denotes significance of relative differences in either toxin changes among the three modalities of dialysis

Table 4 Associations between indoxyl sulfate (IS) and p-cresol sulfate (p-CS) changes after 24 weeks of cycles of all the dialysis modalities employed in this study, i.e., lfHD, hFHD, and HDF, with changes in biochemical indices investigated

		IS			p-CS		
Biochemical indices	n	β	95% Cl for β	*p*	β	95% Cl for β	*p*
Prealbumin (mg/dL)	19	−0.001	−0.29; 0.29	0.995	0.98	0.38; 1.58	0.001
Albumin (g/dL)	19	4.27	−0.26; 8.81	0.065	33.6	17.3; 51.9	<0.001
hsCRP (mg/dL)	19	−0.43	−0.92; −0.06	0.084	−1.50	−2.44; −0.56	0.002
IL-1β (pg/mL)	15	0.07	−0.01; 0.13	0.019	−0.01	−0.55; 0.53	0.972
IL-6 (pg/mL)	15	−0.03	−0.33; 0.27	0.840	0.003	−1.19; 1.20	0.996
TNF-α (pg/mL)	15	−0.09	−0.42; 0.24	0.604	−0.78	−1,19; 0.65	0.284
Insulin (μIU/mL)	19	0.06	−0.02; 0.14	0.115	0.20	−0.10; 0.49	0.185
Leptin (ng/mL)	19	0.03	−0.07; 0.14	0.551	0.23	−0.09; 0.54	0.157
Visfatin(ng/mL)	19	−0.02	−1.27; 1.24	0.979	−2.18	−10.55; 6.18	0.609

hsCRP high-sensitivity C-reactive protein, *IL* interleukin, *TNF-α* tumor necrosis factor-α, *β* beta coefficient of generalized estimating equations (GEE), *95%CI* 95% confidence intervals

depending on the pre- or post-dilution modification amounts to 25–60 L/treatment. In HDF, water removed from the plasma is replenished by infusion of sterile and apyrogenic substitution fluid, while in hfHD the reverse filtration of dialysis fluid occurs inside the dialyzer (Sirich et al. 2017). Modifications of HDF parameters such as elevation of blood or dialysis fluid flow, increase in dialysate area, and more frequent or extended dialysis sessions may all improve the efficiency of removal of serum solutes (Banerjee et al. 2017; Camacho et al. 2016). Fagugli et al. (2002) have demonstrated that the content of protein-bound solutes decreases during HDF on the day-to-day basis. Vanholder et al. (2016) have failed to find any differences in the removal of serum solutes between lfHD and hfHD. There have been no differences noticed between pre- and post-dilution HDF in a study of Meert et al. (2009). In another study, Meert et al. (2011) have noticed that post-dilution HDF removes strongly protein-bound solutes, such as p-CS, better than hfHD does. That, however, has not been confirmed by Krieter et al. (2010) in a study encompassing just eight patients. In the present study, we used a post-dilution method in 19 patients, and we noticed a greater efficiency of p-CS, but not IS, removal during HDF. The discrepancy in the results may likely stem from a different number of patients investigated in various studies.

The ambiguity concerning the survival rate of dialyzed patients may have to do with the ability

to remove toxic substances from the serum. Some of the major studies on the subject, such as the CONTRAST study (714 patients, lfHD vs. HDF) or the Turkish study (782 patients, hfHD vs. HDF) have failed to substantiate the existence of any appreciable differences in mortality between the groups investigated (Ok et al. 2013; Grooteman et al. 2012). In contradistinction, the ESHOL study (906 patients, hfHD vs. HDF) has demonstrated a 30% lower risk of all-cause mortality, and separately also of cardiovascular mortality, in case of HDF (Maduell et al. 2013). The convective volumes were 20.7 L/treatment (CONTRAST study), 19.5 L/treatment (Turkish study), and 23.7 L/treatment (ESHOL study). Patients who did not reach a minimum of 18 L/treatment of connective volume for 2 months of dialysis were usually excluded from those studies. The relationship between the quantity of convective volume obtained during HDF and the survival rate has been discussed for years, but the issue remains contentious as well. A meta-analysis of Mostovaya et al. (2014) reviews 3 randomized trials involving nearly 2400 patients. The analysis puts emphasis on the relation between a greater convection volume achieved in HDF and better treatment and survival outcomes. In the prospective DOPPS study, a 35% reduction in mortality risk has been demonstrated in European patients receiving a minimum of 15 L of substitution fluid *per* HDF treatment, which corresponds to 17 L of convective volume being the sum of ultrafiltration and substitution fluid, compared to the control group treated with standard HD. The result holds fast after controlling for age, comorbidities, urea clearance, and locally employed procedures (Canaud et al. 2006). A nearly 3-year-long prospective observational study of Panichi et al. (2008) has demonstrated that HDF, with increased substitution volume, decreases mortality rate.

The influence of various methods of dialysis treatment on the long-term survival or cardiovascular complications was beyond the scope of the present single-center study, which is a limitation of the study. We used a relatively small volume of substitution fluid of about 14 L/treatment, which could have affected the effectiveness of removal protein-bound solutes. Nonetheless, we believe we have shown that there are no major differences in the serum clearance of IS and p-CS depending on the dialysis modality chosen, from among lfHD, hfHD, and HDF. A single session of each effectively decreases the serum content of both IS and p-CS. The decrease was about twofold grater *per* mass for p-CS, but the effect of leaching out the toxins wore off with the weeks' long dialysis course. We further conclude that a greater inflammation-prone level of hsCRP leads to a greater removal of serum solutes during a single dialysis session. Reversely, a greater protein level may result in a greater solute binding and a decrease in removal.

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Conflicts of Interest The authors declare no conflict of interest in relations to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The project was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Mold Sensitization in Asthmatic and Non-asthmatic Subjects Diagnosed with Extract-Based Versus Component-Based Allergens

S. Kespohl and M. Raulf

Abstract

Asthmatic patients are suspected of having a higher risk of mold sensitization. Thus, precise diagnosis of fungal sensitization is important. Mold allergen extracts are difficult to standardize, but component-resolved allergy diagnosis may be an alternative to replace extract-based tests. In this research, asthmatic and non-asthmatic subjects were studied for their sensitization to *Aspergillus fumigatus* (Asp f), *Cladosporium herbarum* (Cla h), *Penicillium chrysogenum* (Pen ch), *Alternaria alternata* (Alt a), and *Aspergillus versicolor* (Asp v). Extract-based tests were applied using the skin prick test (SPT) and allergen-specific immunoglobulin E (sIgE). Subjects with extract-based sensitization to Asp f or Alt a were further investigated for sIgE response to recombinant (r) single mold allergens. At least one mold sensitization was found in about 50% of asthmatic and non-asthmatics with the most frequent sensitization to Alt a, followed by Pen ch, Asp f, Cla h, and Asp v. Interestingly, sensitization rate to individual mold species was always higher in asthmatics and was only significant for Pen ch. The component-resolved diagnosis with the sum of rAsp f 1 - rAsp f 4 plus rAsp f 6 matched the extract-based results (SPT and/or sIgE) in 50% of asthmatics and 46% of non-asthmatics, whereas, rAlt a 1 covered 59% of asthmatics and 50% non-asthmatics of extract-based Alt a sensitization. In conclusion, individual fungal sensitization rate was higher in asthmatics compared to non-asthmatics. Extract-based tests, especially SPTs, were most sensitive, but component-based tests covered 80% of extract-based serological sensitization to *Alternaria* and *Aspergillus*.

Keywords

Allergen · *Alternaria alternata* · *Aspergillus fumigatus* · Asthma · Component-based diagnosis · Extract-based diagnosis · Mold · Skin prick test · Specific IgE

1 Introduction

Health risks induced by mold exposure include allergen-specific immunoglobulin E (sIgE)-mediated sensitization accompanied with allergic symptoms. Even though mold exposure is ubiquitous in environmental airborne samples, sensitization rates to primarily indoor (e.g., *Aspergillus* sp., *Penicillium* sp.) and outdoor molds (e.g.,

S. Kespohl (✉) and M. Raulf
Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr University Bochum (IPA), Bochum, Germany
e-mail: kespohl@ipa-dguv.de

Cladosporium sp., *Alternaria* sp.) were found to be lower (<10%) compared to other environmental allergen sources, such as pollen, mites, and animal dander (20–30%) (Haftenberger et al. 2013). In both general population and atopic subjects, the most frequent fungal sensitization is seen to *Alternaria* with 2–13%, depending on the test method and patient group (children versus adults). This is followed by *Aspergillus* with 2–10%, *Penicillium* with 5–8%, and *Cladosporium* with 8% (Kespohl and Raulf 2017). In asthmatic patients, the rates of fungal sensitization are clearly increased, with over 20% for *Alternaria*, *Cladosporium*, and *Penicillium*, and are up to 45% for *Aspergillus* (O'Driscoll et al. 2009). A potentially close relationship between asthma and fungal sensitization, known as allergic fungal airway disease (Rick et al. 2016; Denning et al. 2014) or severe asthma and fungal sensitization, has been described in numerous publications (Knutsen et al. 2012; Agarwal and Gupta 2011; Knutsen and Slavin 2011; Denning et al. 2009; O'Driscoll et al. 2009) and was also the subject of the EAACI Task force (Denning et al. 2014). Therefore, diagnosis of fungal sensitization is very important. However, it is known that fungal test extracts used for the diagnostics are extremely variable, due mainly to the heterogeneity of the fungal raw material used for the production of allergen extracts.

Different strains of mold species, as well as different culture conditions are responsible for the heterogenic protein/allergen mixtures (Esch and Codina 2017). A former study comparing mold skin prick test (SPT) has demonstrated that fungal SPT solutions with identical labeling, provided by different suppliers, are highly variable in protein, antigen, and allergen content (Kespohl et al. 2013). A comparison of SPTs with *in vitro* measurements of sIgE indicated that positive test results are obtained more often with SPTs than sIgE measurement. Test concordance of SPT and sIgE was best with SPT solutions containing a high fungal allergen content (Kespohl et al. 2016). Thus, to optimize and standardize fungal test extracts, the option of purified or recombinantly produced allergens may eliminate the variability of naturally produced fungal extracts (Esch and Codina 2017). Importantly, there has been a reduction in commercially available fungal allergen test extracts (Klimek et al. 2015), creating a diagnostic gap which may be filled by single allergen components.

Improvements in the molecular allergy diagnostics have been recently reviewed (Kleine-Tebbe and Jakob 2017), with examples that included increasing test sensitivity by spiking natural allergen extracts with single recombinant allergens and test selectivity by the application of recombinant marker allergens (Huss-Marp et al. 2015). Regarding fungal sensitization, 111 single allergens (including molds, yeast, dermatophytes, and mushrooms) are confirmed and listed in the Allergen Nomenclature (2018) database. Of these, six are commercially available as singleplex and two as components of a multiplex system (Kespohl and Raulf 2017). Specifically, recombinant *Aspergillus fumigatus* allergens are helpful tools for the differentiation of asthmatic patients with allergic bronchopulmonary aspergillosis (ABPA) from *Aspergillus fumigatus*-sensitized asthmatics without ABPA (Kurup et al. 2000; Crameri 1998).

In the current study, sIgE-mediated fungal sensitization rates were investigated in asthmatic versus non-asthmatic subjects with suspected mold sensitization by applying different extract-based test systems (SPT versus sIgE). Furthermore, component-based sIgE tests were compared to extract-based sIgE test results in both groups.

2 Methods

2.1 Study Group and Data Collection

From a former multicenter study (Kespohl et al. 2016), an asthma group and a non-asthma group were reinvestigated. In brief, patients with suspected mold allergy and/or mold exposure were recruited from 13 allergy practices and clinics (12 German and 1 Polish). The inclusion criteria were the anamnestic, self-reported suspicion, or diagnosed mold allergy or mold exposure

and/or mold-induced allergic symptoms. Mold-induced respiratory symptoms could have occurred occupationally, at home, or both. The study consisted of questionnaires, SPTs, and sIgE measurements. Mold exposure was documented by self-reported patient questionnaire asking for visual mold formation (bigger/smaller DIN A4) in living areas, at workplaces, or during recreation.

The group of asthmatics ($n = 81$) included participants with asthma symptoms in the self-reported patient questionnaire, answering *Yes* to the question "Have you ever been diagnosed with asthma?" and reporting additional asthma medication by answering *Yes* to the question "Are you currently taking regular medicines for respiratory problems?" The group of non-asthmatics ($n = 56$) was composed of participants without asthmatic symptoms, answering *No* to the question "Have you ever been diagnosed with asthma?"

Sensitization to *Aspergillus fumigatus* (Asp f), *Cladosporium herbarum* (Cla h), *Penicillium chrysogenum* (Pen ch), *Alternaria alternata* (Alt a), and *Aspergillus versicolor* (Asp v) was tested using extract-based SPTs and sIgE (Kespohl et al. 2016). Component-based allergy diagnostics were exclusively conducted with serological measurement using singleplex recombinant allergen components, one for Alt a: rAlt a 1 and five for Asp f: rAsp f 1-4 plus rAsp f 6.

2.2 Determination of SPT

Mold SPT solutions of Asp f, Cla h, Pen ch, and Alt a were purchased from four different manufacturers: (1) Allergopharma GmbH & Co. KG (Reinbek, Germany), (2) ALK (Hørsholm, Denmark), (3) HAL Allergy (Leiden, Netherlands), and (4) Lofarma (Milano MI, Italy), and the extracts for Asp v were prepared in-house as described earlier (Kespohl et al. 2013). SPT solutions were pricked in duplicate, and test results were calculated as the mean value of both SPT determinations. SPT cut-point was evaluated by the Youden index, taking sIgE measurements as the "positive standard." A mean wheal size $\geq$1.5 mm (right/left arm double testing) was considered positive as described in detail in Kespohl et al. (2016).

2.3 Determination of Serological Parameters

Serum of patients was collected, and mold sensitization was measured with extract-based ImmunoCAPs (ThermoScientific; Uppsala, Sweden) for the following mold species: Asp f (m3), Cla h (m2), Pen ch (m-1), Alt a (m6), and Asp v (Gm25). Rates of sensitization were given in the percentage values. All patients with extract-based sensitization (at least to one of the four SPTs and/or sIgE) to Alt a and/or Asp f were additionally analyzed for component-based sensitization with recombinant (r) allergens as singleplex ImmunoCAPs. In case of SPT and/or sIgE sensitization to Alt a, recombinant major allergen rAlt a 1 (m229) was tested. Asp f-sensitized subjects (SPT and/or sIgE) were tested with five recombinant allergens: rAsp f 1 (m218), rAsp f 2 (m219), rAsp f 3 (m220), rAsp f 4 (m221), and rAsp f 6 (m222) by ImmunoCAP. Specific IgE values $\geq$0.35 kU/L were considered positive, with the measuring range starting at 0.01 kU/L.

2.4 Statistical Analysis

Comparison of diagnostic agreement of extract-based serological test versus component-based test sIgE values was assessed with nonparametric Spearman's correlation coefficient. In case of Asp f, the sum of single Asp f components was correlated with extract-based Asp f values, using GraphPad Prism 7.04. Significance of contingency table analysis regarding fungal sensitization in asthmatic and non-asthmatic subjects was done with two-sided Fisher's exact test. A p-value <0.05 defined statistically significant differences. A commercial statistical package of GraphPad Prism v7.04 was use for the analysis.

3 Results

3.1 Fungal Sensitization in Asthmatics and Non-asthmatic Subjects

Among the 81 subjects with reported asthmatic symptoms plus asthma medication, 56% were shown to have fungal sensitization to at least one of the five tested mold species using SPT and/or sIgE (Fig. 1). Subjects without asthmatic symptoms (non-asthmatics, $n = 56$) but with suspected fungal allergy exhibited a positive reaction to at least one mold species in 52% of the cases. The most prominent fungal sensitization was seen to Alt a, with 42% in asthmatics and 32% in non-asthmatics. Sensitization to Pen ch was just as frequent as sensitization to Alt a among asthmatics, amounting to 41%. However, in non-asthmatics, the rate of Pen ch sensitization was significantly lower (23%) compared to asthmatics. Sensitization to Asp f was present in 32% of asthmatic vs. 23% of non-asthmatics, followed by sensitization rates to Cla h (26% asthmatics vs. 20% non-asthmatics) and to Asp v (21% asthmatics vs. 13% non-asthmatics). The rate of individual sensitization was always higher among the asthmatic subjects compared to the non-asthmatics but significant only for Pen ch sensitization.

Differences in the applied extract-based diagnostic tests were investigated for SPT and sIgE in asthmatics and non-asthmatics (Fig. 2). SPT detected mold sensitization more frequently compared to sIgE tests using ImmunoCAP, independent of asthma symptoms. With SPTs, 52% of asthma and non-asthmatic subjects showed positive skin reactions to at least one mold species in at least one of the four applied SPT extracts, whereas 37% of asthmatics and 25% of non-asthmatics were tested positive to at least one mold species using the serological sIgE test with ImmunoCAPs. Considering the single SPT results according to individual manufacturers, it was seen that the rate of mold sensitization was lower using only one SPT compared to the combined. However, discrepancy between mold sensitization measured by SPT and sIgE was still there. For asthmatics, the rate of fungal sensitization to at least one mold species was shown to be between 33% and 47% with different SPTs compared to 37% with sIgE. In non-asthmatics, 38–46% showed positive skin reactions in SPTs and only 25% with sIgE. Nevertheless, the

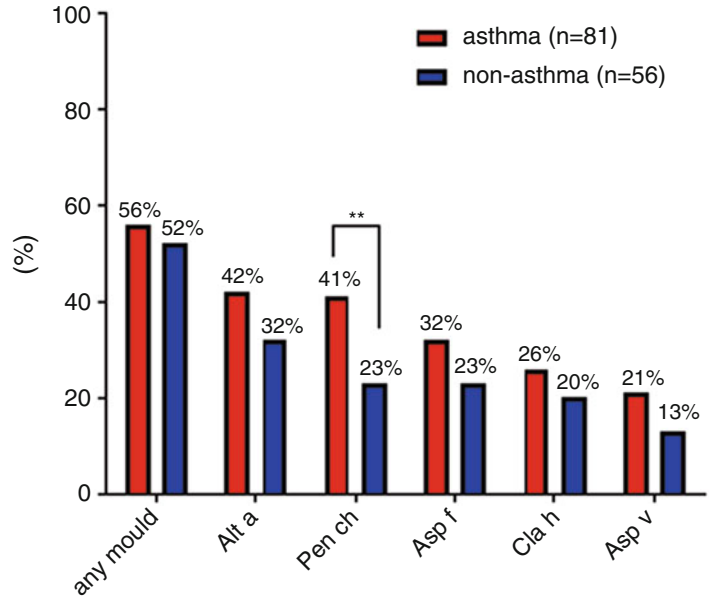

Fig. 1 Extract-based fungal sensitization diagnosed by skin prick test (SPT) and/or allergen-specific immunoglobulin E (sIgE) in asthmatic and non-asthmatic subjects; $^{**}p < 0.01$

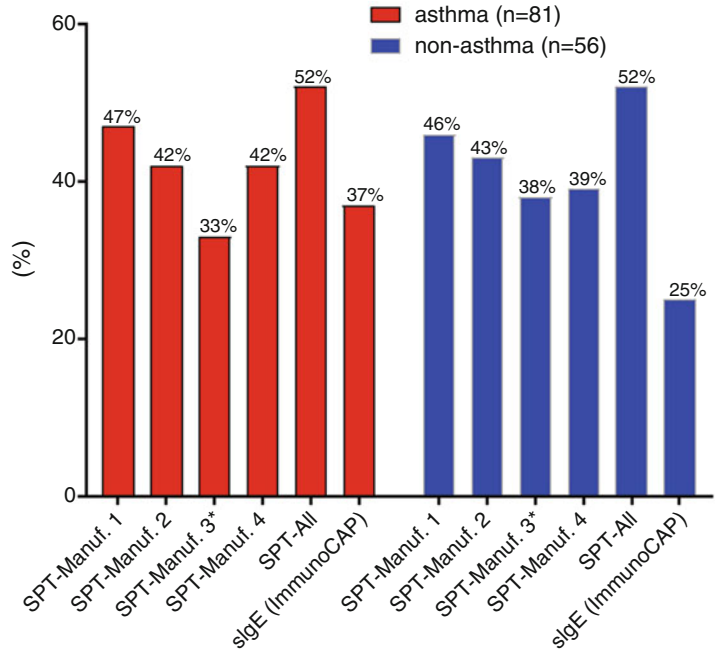

Fig. 2 Sensitization rate to at least one mold species, diagnosed by skin prick test (SPT) against *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *and Penicillium chrysogenum* from four different manufacturers (Manuf. 1–4) or diagnosed by allergen-specific immunoglobulin E (sIgE) measured with the corresponding ImmunoCAPs in asthmatic and non-asthmatic subjects. *Manufacturer offered no *Claosporium herbarum* SPT solution

concordance of positive SPT and positive sIgE was always higher in asthmatics than in non-asthmatics (Table 1). The best concordance was seen for Alt a with 71% positive SPT plus sIgE in asthmatics and 56% in non-asthmatics. For all other mold species, the test concordance was below 50% with a range of difference from 2% to 25% in asthmatics compared to non-asthmatics (Table 1). The highest rate of discrepancy in SPT/sIgE concordance (32%) was seen with Pen ch sensitization, with 8% in non-asthmatics compared to 40% in asthmatics. Only this high discrepancy of concordance between asthmatic and non-asthmatics was significant.

3.2 Component-Based Versus Extract-Based sIgE Testing

Among the studied subjects, sensitization to Alt a was found to be most common, including 34/81 (42%) asthmatics and 18/56 (32%) non-asthmatics (Fig. 1), as diagnosed with both SPT and sIgE. Serological sensitization was measured in 26 of the 34 Alt a-sensitized asthmatics by means of sIgE concentration $\geq$ 0.35 kU/L and in 11 of 18 Alt a-sensitized non-asthmatics (Table 1). The concordance of SPT and sIgE was between 71% in asthmatics and 56% in non-asthmatics. A component-based test for Alt a was conducted with the major allergen, rAlt a 1, using singleplex ImmunoCAP in all subjects who exhibited *Alternaria* sensitization (SPT and/or sIgE) (Fig. 3a and b). With component-based rAlt a 1 measurement, no additional sensitization was detected that was not detected with the extract-based sIgE testing. Specific IgE concentration to component rAlt a 1 $\geq$ 0.35 kU/L was measured in 20 asthmatics (Fig. 3a), all of whom also had sIgE concentration $\geq$ 0.35 kU/L to the allergen extract Alt a. Therefore, taking only the serological tests into account, rAlt a 1, when used as a component-based tool, detected 77% (20/26) of extract-based serological sensitization in asthmatics. Nevertheless, eight subjects with Alt a sensitization diagnosed solely by SPT were not detected using the component-based rAlt a 1.

In the group of non-asthmatics, similar results were obtained (Fig. 3b). Here, component-based

Table 1 Extract-based mold sensitization rate and concordance of allergen-specific immunoglobulin E (sIgE) and skin prick test (SPT) in asthmatics and non-asthmatics

Patient group	Mold allergen	sIgE (+) (n)	SPT (+) (n)	SPT (+) and/or sIgE (+) (n)	SPT (+) and sIgE (+) (n)	SPT (+) and sIgE (−) (n)	SPT (−) and sIgE (+) (n)	Concordance SPT and sIgE (%)
Asthma	Alt a	26	32	34	24	8	2	71
Non-asthma	Alt a	11	17	18	10	7	1	56
Asthma	Asp f	13	24	26	11	13	2	42
Non-asthma	Asp f	6	11	13	4	7	2	30
Asthma	Pen ch	23	40	45	18	22	5	40
Non-asthma	Pen ch	1	11	12	1	11	0	8
Asthma	Cla h	9	21	21	9	12	0	43
Non-asthma	Cla h	2	11	11	2	9	0	18
Asthma	Asp v	3	16	17	2	13	1	12
Non-asthma	Asp v	1	7	7	1	6	0	14

Alt a Alternaria alternata allergen, *Asp f Aspergillus fumigatus* allergen, *Pen ch Penicillium chrysogenum* allergen, *Cla h Cladosporium herbarum* allergen, *Asp v Aspergillus versicolor* allergen

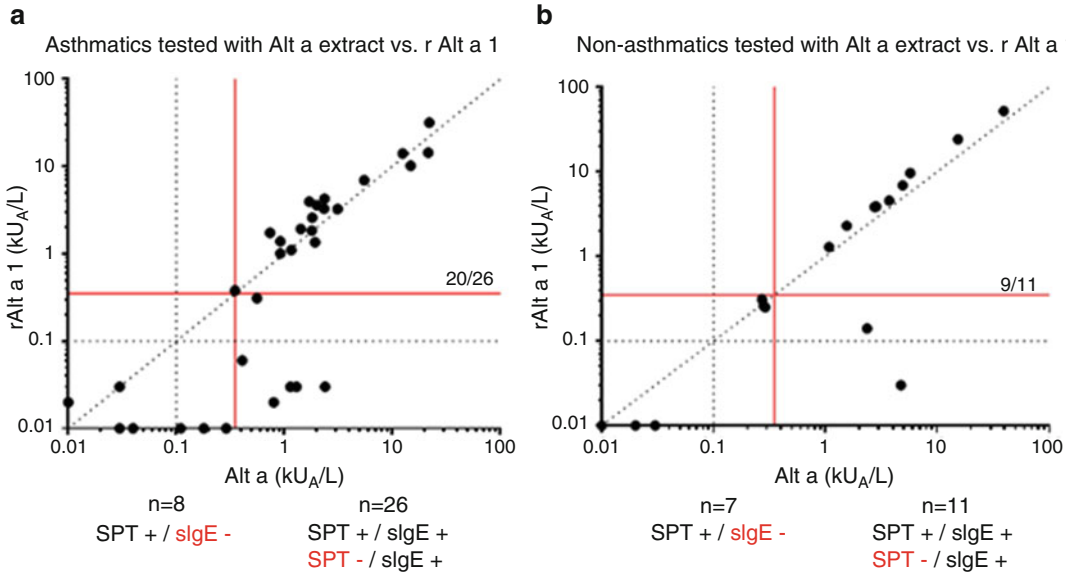

Fig. 3 Comparison of extract-based vs. component-based allergen-specific immunoglobulin E (sIgE) sensitization with *Alternaria alternata* in asthmatic (**a**) and non–asthmatic (**b**) subjects

sensitization to rAlt a 1 was seen in nine non-asthmatic subjects all of whom reacted positively to extract-based Alt a in SPT and/or sIgE. Calculating only serological Alt a sensitization, component-based rAlt a 1 measured positive sIgE values ($\geq$ 0.35 kU/L) in 9 out of the 11 (82%) extract-based positive non-asthmatic subjects. Of note, patients with Alt a sensitization

in SPT without sIgE to Alt a were not detected by rAlt a 1. In both groups, single allergen rAlt a 1 met the criteria for a major allergen and was recognized by more than 50% of subjects. Nonparametric Spearman's correlation was highly significant ($p < 0.0001$) among both asthmatics ($r = 0.871$) and non-asthmatics ($r = 0.863$).

In comparison to Alt a, Asp f sensitization with extract-based tools (SPT and/or sIgE) was seen in 26 asthmatics and 13 non-asthmatics (Table 1). For component-based diagnostic tools, five single allergens rAsp f 1, rAsp f 2, rAsp f 3, rAsp f 4, and rAsp f 6 were tested. There was no additional sIgE sensitization detected by component-based testing compared to extract-based tests, neither in asthmatics nor in non-asthmatics (Fig. 4a and b). Specific IgE to single components was exclusively detected in subjects with sIgE sensitization to Asp f (extract-based ImmunoCAPs). Specific IgE to allergen components rAsp f 1 and rAsp f 3 was most frequently measured in about 50% of both groups – in 6 out of the 13 asthmatics and in 4 (rAsp f 1) and 3 (rAsp f 3) out of the 6 non-asthmatics (Table 2). *Aspergillus* allergens, rAsp f 2 and rAsp f 4, each tested positively in five subjects and rAsp f 6 in three subjects from both groups. Taken together, the component-based allergen pattern for *Aspergillus* sensitization was comparable in asthmatics and non-asthmatics. For a comparison of extract-based serological sensitization with component-based tests, the sum of all single components (rAsp f 1–Asp f 6) was taken. In asthmatics, 10 out of the 13 (77%) subjects with extract-based serological Asp f

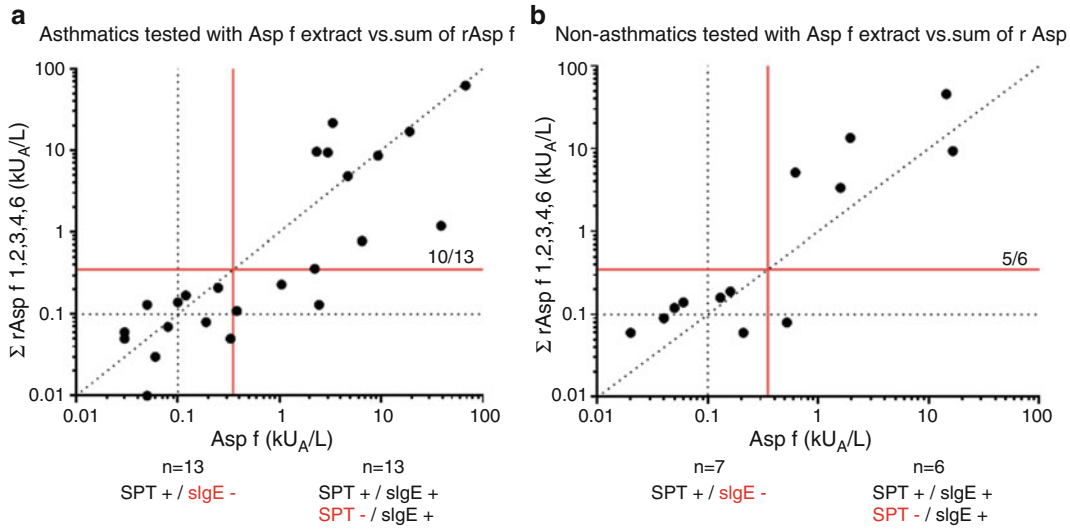

Fig. 4 Comparison of extract-based vs. sum of component-based allergen-specific immunoglobulin E (sIgE) sensitization with *Aspergillus fumigatus* in asthmatic (**a**) and non–asthmatic subjects (**b**)

Table 2 Rate of sensitization against single aspergillus (rAsp f) allergens in asthmatics and non-asthmatics with allergen-specific immunoglobulin E (sIgE) and skin prick test (SPT)

Allergen	All Asp f sensitized	Asthmatics	Non-asthmatics
rAsp f 1	10/19 = 53%	6/13 = 46%	4/6 = 67%
rAsp f 2	5/19 = 26%	4/13 = 31%	1/6 = 17%
rAsp f 3	9/19 = 47%	6/13 = 46%	3/6 = 50%
rAsp f 4	5/19 = 26%	4/13 = 31%	1/6 = 17%
rAsp f 6	3/19 = 16%	2/13 = 15%	1/6 = 17%

Asp f Aspergillus fumigatus allergens

sensitization were also positive in the component-based tests (Fig. 4a). In non-asthmatic subjects, five out of the six (83%) tested positive to the sum of single rAsp f components (Fig. 4b). Correlation of extract-based vs. sum of component-based tests for Asp f sensitization was significant in both groups ($p < 0.005$), with $r = 0.850$ for asthmatics and $r = 0.770$ for non-asthmatics.

4 Discussion

The current study compared mold sensitization in asthmatics and non-asthmatics with extract-based allergy tests applied as SPT and as serological sIgE measurements. The results showed that fungal sensitization rate to at least one of the five tested mold species was comparable – with 56% in asthmatics and 52% in non-asthmatics. A high mold sensitization rate revealed here was consistent with a previous survey in which the mold sensitization rate was 66% in asthmatics (O'Driscoll et al. 2009). But for non-asthmatics, more than 50% fungal sensitization observed in the current study was unexpectedly high. This could be due to the recruiting criteria for patients, which only included subjects with reported mold exposure or suspected mold allergy, who were obviously more sensitized compared to the general population.

For the five individual mold species tested, it was shown that sensitization was always higher in asthmatics compared to non-asthmatics. The most frequent mold sensitization seen in both groups (42% in asthmatics and 32% in non-asthmatics) was to *Alternaria*. The dominant role of *Alternaria* as a source of IgE-mediated sensitizer and inducer of respiratory symptoms (asthma, wheeze, or allergy) has been reported in several studies and case reports (Behbod et al. 2015; Salo et al. 2006; Bush and Prochnau 2004; Zureik et al. 2002). Sensitization rates against *Aspergillus*, *Cladosporium* and *Penicillium* in non-asthmatics also were with >20% higher in the current study compared to former studies in the general population and in patients with allergic problems (Haftenberger et al. 2013; Gent et al. 2012; Szewzyk et al. 2011; Heinzerling et al. 2009). Of note, there was a significantly higher sensitization rate to Pen ch in asthmatics (41%) compared to non-asthmatics (23%). Former studies have shown significantly higher odds ratio of asthma in relation to specific IgE to *Aspergillus* and *Cladosporium* (Jaakkola et al. 2006) or *Alternaria* (Zureik and Orehek 2002), but not for *Penicillium* in adult asthmatics. Nevertheless, there are neonate cohorts or studies in children indicating that *Penicillium* is significantly associated with respiratory effects among the sensitized children (Caillaud et al. 2018; Gent et al. 2012; Rosenbaum et al. 2010; Bundy et al. 2009). Therefore, *Penicillium* sensitization should be considered regarding fungal sensitization in asthmatics.

Among the extract-based test systems, SPT revealed mold sensitization with >50% higher to at least one mold species. This was more than what was observed with serological sIgE tests with 25% in non-asthmatics and 37% in asthmatics. These results may be due to the fact that in the current study, positive SPT results were generated from skin tests using four different SPT solutions, from different suppliers, all tested as double values. When calculating the sensitization rate of only one SPT solution with sIgE, it was shown that it was higher in SPT compared to sIgE only if SPT solutions with high antigen/allergen content were used (Kespohl et al. 2016). In contrast, if SPT solution had a low antigen/allergen content, the sIgE-based sensitization rate was higher in comparison to SPT. Discordant fungal sensitivity, as diagnosed by SPT or sIgE, has been investigated previously by O'Driscoll et al. (2009) who report 77% concordance of SPT and sIgE for fungal sensitization to at least one mold species, with slightly more positive sIgE tests. Further, double skin prick testing improves test reproducibility enormously, especially in extracts without major allergens, such as many occupational and mold allergens (van Kampen et al. 2013). In addition, SPT results depend on the arm position used, as wheal size closer to the elbow is slightly larger than that at the wrist (Kespohl et al. 2016).

Component-based allergy diagnosis has been introduced to resolve the issue of standardization

in fungal allergen test solutions, thereby increasing test sensitivity and selectivity (Kleine-Tebbe and Jakob 2015). In the current study, rAlt a 1 was used as a singleplex for *Alternaria* sensitization and was detected in about 80% of Alt a extract-based serological sensitization, independent of asthmatic symptoms. For *Alternaria*, component-based diagnosis was comparable with extract-based serological diagnosis, since rAlt a 1 was the major allergen available in sufficient amounts in the *Alternaria* test extracts used. That is in line with the result of a former study by Asturias et al. (2005) showing that *Alternaria* diagnostics could be substituted for by natural or recombinant Alt a 1 that was recognized in 98% of 42 patients with positive SPT and serology to Alt a. Another study by Vailes et al. (2001) has found sIgE response to rAlt a 1 in 93% of patients with asthma/rhinitis, exhibiting sIgE concentration (> CAP-class 2) to *Alternaria* extract. Other patients, such as those with atopic dermatitis or cystic fibrosis, with sIgE concentration (> CAP-class 2) to *Alternaria*, were less frequently sensitized to single allergen rAlt a 1, in 47% and 60%, respectively.

Aspergillus fumigatus component-based diagnosis has been intensively investigated (Crameri 1998), with a specific goal to differentiate between allergic/asthmatics and patients with allergic bronchopulmonary aspergillosis (ABPA) or cystic fibrosis. A combination of sIgE sensitization to rAsp 2 plus rAsp f 4 plus rAsp f 6 appears significant enough to discriminate between asthmatics and patient with ABPA (Kurup et al. 2000). In the current study, single component-based sIgE diagnosis using singleplex rAsp f 1, rAsp f 2, rAsp f 3, rAsp f 4, and rAsp f 6 assessed in 10 out of the 26 Asp f (extract-based)-sensitized asthmatics and in 5 out of the 13 non-asthmatics resulted in a positive response ($\geq$ 0.35 kU/L) to the sum of all five rAsp f components (Table 2). There was no difference in sIgE sensitization profile or frequency to any of the tested single Asp f allergens depending on asthma or non-asthma status. In both groups, rAsp f 1 and rAsp f 3 were the most frequently detected allergens. A previous study by Crameri (1998) also shows rAsp f 1 and rAsp f 3 to be the most prominent allergens in allergic, asthmatic and ABPA patients. A reported frequency of up to 70% sensitization to single rAsp f components was not confirmed in the current study, taking all (SPT and/or sIgE) Asp f-sensitized subjects into account. Here, none of the tested single rAsp f allergens exceeded the 26% sensitization rate among *Aspergillus*-sensitized subjects. However, calculating the sensitization rate to single rAsp f allergens on the basis of subjects with sIgE to Asp f resulted in up to 53% sensitization to rAsp f 1 and 47% to rAsp f 3.

In conclusion, it was shown that extract-based diagnosis conducted as skin prick test was the most sensitive and was therefore recommended as the first choice. Serological sensitization to *Alternaria* was measured by rAlt a 1 in 78% of all patients and could substitute for the extract-based serological measurement. For serological *Aspergillus* sensitization, all five rAsp f single allergens were necessary to account for 80% of the extract-based sIgE sensitization. Further research is necessary to identify clinically relevant allergens, especially marker allergens; for instance, serine proteases may be relevant for diagnostic purposes in fungal allergy.

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Conflicts of Interest The authors declare no conflict of interest in relation to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The Ethics Committee of the Medical Faculty of the Ruhr-University Bochum in Germany approved the implementation of all necessary examinations (register no. 4104-11).

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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Fructose Consumption and Lipid Metabolism in Obese Children and Adolescents

Aneta Czerwonogrodzka-Senczyna, Małgorzata Rumińska, Anna Majcher, Dominika Credo, Anna Jeznach-Steinhagen, and Beata Pyrżak

Abstract

Inappropriate dietary habits influence the development of excessive body weight. The role of added sugars, including fructose, notably is significant in this process. It is estimated that fructose intake has increased many times over the past two centuries. The aim of the study was to define the effect of fructose consumption on anthropometric indices and lipid metabolism in obese (body mass index (BMI) >30 kg/m^2) children and adolescents. The study included 84 patients (47 girls and 37 boys) aged 7–18 years, divided into prepubertal, pubertal, and postpubertal age groups. Aside from BMI, the assessment comprised waist circumference, body composition estimated with bioelectrical impedance (BIA), plasma lipid profile, fructose intake consumption based on a 3-day menu analysis, and a number of calculated atherogenic indices. The major findings were that total daily fructose intake was high, on average, ranging from 19 to 26 g, with no appreciable relation to age. A higher fructose intake from beverages is significantly associated with the percentage of body fat, waist circumference, waist-to-height ratio, and also with the content of total cholesterol, triglycerides, and the level of atherogenic indices. In conclusion, fructose appears a particularly unfavorable component in children's diet as it is conducive to visceral obesity and atherogenic lipid profile. However, inadequate proportions of other macronutrients may also be at play in the development of metabolic diet-related disorders.

Keywords

Adolescents · Anthropometry · Children · Diet · Fructose · Lipid metabolism · Nutrition · Obesity

1 Introduction

Inappropriate dietary habits constitute one of the main factors influencing the development of excessive body weight and obesity (Lanfer et al. 2010). Individual components of the diet play a significant role in maintaining normal body weight (Wojtyła-Buciora et al. 2015; Kapka-Skrzypczak et al. 2012; AHA 2011). The most

A. Czerwonogrodzka-Senczyna (✉) and A. Jeznach-Steinhagen
Department of Clinical Dietetics, Warsaw Medical University Warsaw, Warsaw, Poland
e-mail: aneta.czerwonogrodzka@wum.edu.pl

M. Rumińska, A. Majcher, D. Credo, and B. Pyrżak
Department of Pediatrics and Endocrinology, Warsaw Medical University Warsaw, Warsaw, Poland

common nutritional errors among Polish adolescents are the following: improper number of meals, skipping breakfast, eating snacks between meals and at night, and general overeating leading to excess dietary energy consumption relative to energy expenditure, all of which result in weight increase. Fat delivered with food is stored in adipocytes, but also excessive amounts of carbohydrates and proteins may be converted into fatty acids in the liver (Mahan et al. 2011).

Recently, increasing attention has been paid to the influence of carbohydrates on the development of obesity. That includes sugars and sweeteners added while eating and during the food production process, such as white or brown sugar, corn syrup, fructose syrup, honey, molasses, crystalline dextrose, maple syrup, sucrose, glucose, maltose, lactose, or inverted sugar (USDA 2015a). Fructose is one of the most common carbohydrates added as a sweetener in juices, candied fruit, ice cream, yoghurt, desserts, and alcoholic beverages. It also is a natural sugar present in fruits, juices, and honey. The main source of fructose in unprocessed products is sucrose. It is suggested that sucrose and fructose influence metabolic processes in a similar way. Therefore, similar disorders may develop due to overconsumption of excessive amounts of table sugar and corn syrup that contain substantial amounts of fructose (Jang et al. 2018).

A high-fructose diet is responsible not only for adipocyte accumulation in the visceral tissue but also for ectopic fat depositions, e.g., in muscles or liver, caused by enhanced hepatic lipogenesis. Such a diet causes unfavorable changes in the blood lipid profile, leading to increased content of low-density lipoprotein cholesterol (LDL) and triglycerides (TG), which may exceed the relative increase in glucose itself. There is a concomitant reduction in high-density lipoprotein cholesterol (HDL), all of which is conducive to the development of atherosclerosis and cardiovascular diseases (Mahan et al. 2011). Epidemiologic research indicates a strong correlation between high dietary fructose and obesity, nonalcoholic fatty liver disease, type 2 diabetes, renal dysfunction, or cardiovascular disease (Caliceti et al. 2017; Jegatheesan and De Bandt 2017; Macdonald 2016; Bravo et al. 2013; Johnson et al. 2013; Chong et al. 2007). However, the underlying mechanisms of that correlation remain unclear.

It is estimated that fructose consumption has increased 100-fold over the last two centuries. It currently amounts to about 10% energy value of the diet in the USA (Marriott et al. 2009). Fructose consumption is highest in adolescents aged 12–18 and provides, on average, 12% energy, with 25% of adolescents consuming more than over 15% energy from fructose (Bray 2010). Part of the confusion surrounding fructose stems from the lack of precise recommendations concerning its consumption. Therefore, this study seeks to define the effect of fructose consumption on anthropometric and lipid metabolic indices in obese children and adolescents.

2 Methods

The study included 84 patients (47 girls and 37 boys) hospitalized in the Department of Pediatrics and Endocrinology of Warsaw Medical University in Warsaw, Poland. All of the patients met the body mass index (BMI) criteria of obesity, defined by the WHO and Polish national standards (Kułaga et al. 2015). Patients were divided into three groups regarding the stage of their biological development:

- prepubertal, aged 7–12 years (n = 27)
- pubertal, aged 13–15 years (n = 25)
- postpubertal, aged 16–18 years (n = 32)

The following parameters were measured: body weight (kg), height (cm), and waist circumference (cm), and BMI and waist-to-height ratio (WHR) were calculated. BMI was standardized according to WHO guidelines and presented as BMI z-score (de Onis and Blössner 2003). WHR $\geq$ 0.5 was adopted as the criterion of abdominal obesity (McCarthy and Ashwell 2006). Body composition was evaluated with a bio-impedance method using a Maltron Body Fat Analyzer BF–905 (Health Professional;

Solutions, Alderley, Australia). The adipose tissue percentage (%FAT) was referred to normal values of 19% in females and 15% in males.

A profile of blood lipids, consisting of total cholesterol (TC), high-density lipoprotein cholesterol (HDL), and triglycerides (TG), was evaluated with a calorimetric enzymatic method using a VITROS 5600 analyzer (Ortho Clinical Diagnostic; Raritan, NJ). Low-density lipoprotein cholesterol (LDL) was calculated with the Friedewald formula. The upper limits of normal values were taken as the following: TC $\geq$200 mg/dL, LDL $\geq$130 mg/dL, and TG $\geq$100 mg/dL. The HDL level $\geq$ 45 mg/dL was considered as the norm. The following indices of atherogenicity were calculated: non-HDL (TC-HDL), TC/HDL, and TG/HDL. Abnormal values of these indices were taken as those set in a study of Rumińska et al. (2012) and were $\geq$ 123 for non-HDL, $\geq$3.5 for TC/HDL, and $\geq$ 4.0 for TG/HDL.

The evaluation of nutritional habits of patients was based on an interview covering recent 3 days, including 1 weekend day. Food portion size was assessed with the help of a photo album presenting food products and dishes, adopted from a study of Szponar et al. (2000). Dietary fructose content was calculated on the basis of Finnish tables of fructose content in alimentary products (NIHW 2017). Products containing less than 0.1 g of fructose were viewed as fructose-free. Data obtained were compared against the reference values for food ingredients consumption in the Polish population according to age and gender (Jarosz 2017).

Continuous data were presented as means $\pm$SD and min–max range, while categorical data as frequency and percentages. Inter group differences were evaluated with one-way ANOVA. When the null hypothesis of no differences between groups was rejected, data were subjected to Fisher's least significant difference (F-LSD) analysis to make direct comparisons between two means, i.e., each group to every other group. A difference exceeding the LSD value was considered a significant result. The analysis included 95% confidence intervals (95%CI) for means and Pearson's correlation coefficients. A p-value <0.05 defined statistically significant differences. The analysis was conducted with a commercial package of IBM SPSS Statistics v23 (IBM Corp; Armonk, NY).

3 Results

3.1 Anthropometric Indices

Overall, anthropometric indices were smallest in the youngest children, aged 7–12, with BMI (26.4 $\pm$ 2.9 vs. 33.7 $\pm$ 5.2), waist circumference (82.5 $\pm$ 7.7 cm vs. 99.9 $\pm$ 12.5 cm), and adipose tissue content (%FAT) (35.8 $\pm$ 5.7% vs. 40.4 $\pm$ 8.4%) being significantly less, compared to the oldest group aged 16–18 ($p < 0.001$ for all). BMI in all children older than 12 met the criteria of obesity in comparison even with reference values for adults (>30 kg/m^2) (Table 1).

3.2 Macronutrient and Energy Intake

The mean caloric value of the diet exceeded the recommended daily allowance (RDA) only in the youngest group of children by 8.2%. Menu comparison of the children aged 7–12 and 13–15 against the reference values showed that the upper limit of RDA was exceeded concerning the intake of protein, on average, by 21.2% and 9.7%, fat by 39.0% and 11.2%, and sucrose by 67.3% (15.5% of daily energy supply) and 20.2% (13% of energy supply), respectively. Energy and macronutrient intake was lowest in the oldest group compared to the youngest group of children; a decline achieved significance in case of protein and sucrose intake ($p < 0.05$) (Table 2).

Children from the youngest age group consumed, on average, less fructose from sweetened beverages and fruits, which tended to decrease fructose-related daily energy intake. However, the differences among the age groups remained statistically insignificant due to a rather wide spread of data (Table 3).

Table 1 Anthropometric indices in age groups

Variable		7–12 years (n = 27)	13–15 years (n = 25)	16–18 years (n = 32)	ANOVA + F–LSD
					F(2,81) = 22.70; p < 0.001
BMI (kg/m^2)	Mean ± SD	26.4 ± 2.9	32.9 ± 4.7	33.7 ± 5.2	1 vs. 2; p < 0.001
	Min–Max	21.7–34.1	26.2–44.9	27.1–44.0	1 vs. 3; p < 0.001
	95%CI	25.3–27.6	30.9–34.8	31.8–35.6	2 vs. 3; p = 0.514
					F(2,81) = 1.62; p = 0.204
BMI z-score	Mean ± SD	2.9 ± 0.8	4.6 ± 1.8	4.8 ± 1.9	
	Min–Max	1.9–4.9	2.2–9.2	2.1–8.8	
	95%CI	2.7–3.3	3.9–5.4	4.1–5.5	
					F(2,81) = 26.44; p < 0.001
Waist circumference (cm)	Mean ± SD	82.5 ± 7.7	99.6 ± 9.1	99.9 ± 12.5	1 vs. 2; p < 0.001
	Min–Max	69.0–100.0	83.0–120.0	80.0–127.0	1 vs. 3; p < 0.001
	95%CI	79.5–85.6	95.9–103.4	95.4–104.4	2 vs. 3; p = 0.913
					F(2,81) = 2.00; p = 0.141
WHR	Mean ± SD	0.56 ± 0.03	0.59 ± 0.06	0.58 ± 0.06	
	Min–Max	0.51–0.66	0.47–0.76	0.50–0.70	
	95%CI	0.55–0.58	0.57–0.62	0.56–0.61	
					F(2,81) = 6.30; p = 0.003
%FAT	Mean ± SD	35.8 ± 5.7	40.7 ± 8.5	40.4 ± 8.4	1 vs. 2; p = 0.009
	Min–Max	28.2–51.3	28.6–58.2	20.0–52.6	1 vs. 3; p = 0.001
	95%CI	33.6–38.1	37.1–44.3	37.4–43.4	2 vs. 3; p = 0.607

Statistical elaboration with one-way ANOVA followed by Fisher's least significant difference (F-LSD)
BMI body mass index, *WHR* waist-to-height ratio, *%FAT* percent of body fat, *95%CI* 95% confidence intervals

3.3 Serum Lipid Profile

Abnormal values of TC concentration (≥ 200 mg/dL) were present in 14 (17%) children, LDL (≥ 130 mg/dL) in 15 (18%), HDL (< 45 mg/dL) in 62 (74%), and TG (≥ 100 mg/dL) in 58 (69%), and increases in the atherogenic indices of non-HDL (≥ 123) were in 49 (58%), TC/HDL (> 3.5) in 67 (80%), and TG/HDL (> 4) in 30 (36%) children. Significant differences were observed between the 7–11 and 12–15 years of age as regards the mean content of TG and the atherogenic non-HDL and TG/HDL (Table 4).

3.4 Pearson's Analysis of Associations Between Variables

Associations between consumption of fructose from various sources and anthropometric variables were assessed using Pearson's coefficient as a measure of linear relationship between pairs of variables. Consumption of fructose contained in beverages significantly correlated with waist circumference ($r = 0.192$; p = 0.019), WHR ($r = 0.174$; p = 0.030), and % FAT ($r = 0.186$; p = 0.023). Children who consumed more dietary fructose also had a tendency toward a higher waist circumference, WHR, and % body fat (Table 5).

A higher total fructose consumption was associated with a tendency toward a higher concentration of TC ($r = 0.122$; p = 0.088), TG ($r = 0.124$; p = 0.083), and non-HDL ($r = 0.121$; p = 0.093). The atherogenic index TG/HDL ($r = 0.153$; p = 0.046) was associated with a higher dietary intake of fructose (p = 0.046). Associative trends were demonstrated between fructose taken in from beverages and TC (r = 0.141; p = 0.059), TG (r = 0.127; p = 0.078), non-HDL (r = 0.136; p = 0.068), and TG/HDL (r = 0.138; p = 0.064) (Table 6).

Table 2 Energy consumption and macronutrients in age groups

Variable		7–12 years (n = 27)	13–15 years (n = 25)	16–18 years (n = 32)	ANOVA + F–LSD
					F(2,81) = 2.79; p = 0.062
Energy (kcal/day)	Mean ± SD	1732 ± 589	1800 ± 604	1480 ± 431	
	Min–Max	971–3426	803–3356	796–2611	
	95%CI	1499–1964	1551–2050	1325–1636	
	Reference value	1600	1950	2300	
					F(2,81) = 4.88; p = 0.010
Protein (g/day)	Mean ± SD	60.6 ± 17.7	66.9 ± 18.9	52.8 ± 15.0	1 vs. 2; p = 0.193
	Min–Max	35.1–116.0	34.8–107.3	19.1–90.0	1 vs. 3; p = 0.081
	95%CI	53.7–67.6	59.1–74.7	47.4–58.2	2 vs. 3; p = 0.003
	Reference value	50	61	81	
					F(2,81) = 2.11; p = 0.128
Fat (g/day)	Mean ± SD	68.1 ± 33.4	66.7 ± 28.9	54.2 ± 24.2	
	Min–Max	35.9–191.6	17.7–125.9	3.7–110.0	
	95%CI	54.9–81.3	54.8–78.7	45.5–62.9	
	Reference value	49	60	70	
					F(2,81) = 1.74; p = 0.183
Carbohydrates (g/day)	Mean ± SD	236.8 ± 79.2	252.2 ± 99.3	212.1 ± 69.1	
	Min–Max	104.9–442.6	117.8–588.7	78.7–349.5	
	95%CI	205.5–268.1	211.2–293.2	187.2–237.0	
	Reference value	240	292.5	345	
					F(2,81) = 3.31; p = 0.041
Sucrose (g/day)	Mean ± SD	66.9 ± 37.2	58.9 ± 33.4	43.6 ± 35.8	1 vs. 2; p = 0.417
	Min–Max	10.3–160.1	10.5–170.5	3.3–129.6	1 vs. 3; p = 0.014
	95%CI	52.2–81.7	45.1–72.7	30.6–56.5	2 vs. 3; p = 0.110
	Reference value	40	49	58	
					F(2,81) = 0.80; p = 0.452
Fiber (g/day)	Mean ± SD	18.9 ± 7.3	20.7 ± 7.2	18.2 ± 7.9	
	Min–Max	8.0–40.6	5.1–30.0	9.6–47.2	
	95%CI	16.0–21.8	17.7–23.6	15.3–21.0	
	Reference value	16	19	21	

Statistical elaboration with one-way ANOVA followed by Fisher's least significant difference (F-LSD)
RDA recommended daily allowance

4 Discussion

Improper diet, particularly during growth and development, involving an excessive caloric intake and inadequate content of some nutrients, may influence health condition. Childhood overweight and obesity increase the risk of disorders of glucose homeostasis but also of cardiovascular disorders or abdominal malignancy in adulthood. The risk of developing gout in men and arthritis or femoral bone fractures in women also is increased. It has been demonstrated that marked obesity during early adulthood shortens the predicted lifespan by about 13 years and increases the general mortality rate (Hagman et al. 2014; Milona et al. 2014). The development of metabolic and hemodynamic disorders is significantly

Table 3 Fructose intake *per* day in age groups

Variable		7–11 years (n = 27)	12–15 years (n = 25)	16–18 years (n = 32)	ANOVA + F–LSD
					F(2,81) = 1.62; p = 0.205
Total fructose (g)	Mean ± SD	19.6 ± 13.3	26.1 ± 17.9	18.9 ± 16.6	
	Min–Max	5.4–60.7	5.3–81.2	0.4–72.3	
	95%CI	14.4–24.9	18.7–33.5	12.9–24.9	
					F(2,81) = 0.73; p = 0.485
Energy from fructose (%)	Mean ± SD	4.6 ± 2.6	5.9 ± 4.0	5.20 ± 4.4	
	Min–Max	1.2–10.3	1.6–20.5	0.1–19.7	
	95%CI	3.6–5.7	4.3–7.5	3.6–6.8	
					F(2,81) = 0.20; p = 0.821
Fructose in vegetables (g)	Mean ± SD	8.5 ± 7.8	6.9 ± 8.1	7.4 ± 11.4	
	Min–Max	0.0–31.1	0.0–38.2	0.0–65.4	
	95% CI	5.4–1.6	3.6–10.3	3.3–11.5	
					F(2,81) = 1.02; p = 0.364
Fructose in fruits (g)	Mean ± SD	2.9 ± 1.5	3.9 ± 3.9	3.2 ± 1.7	
	Min–Max	0.5–5.6	0.0–21.0	0.0–6.5	
	95%CI	2.3–3.5	2.3–5.5	2.6–3.8	
					F(2,81) = 1.81; p = 0.170
Fructose in beverages (g)	Mean ± SD	6.4 ± 11.3	13.4 ± 18.2	7.4 ± 13.0	
	Min–Max	0.0–54.9	0.0–60.0	0.0–61.0	
	95%CI	1.9–10.8	5.8–20.9	2.7–12.1	
					F(2,81) = 1.60; p = 0.208
Fructose in other sources (g)	Mean ± SD	1.8 ± 2.7	1.3 ± 1.2	0.9 ± 1.3	
	Min–Max	0.1–14.2	0.0–4.0	0.0–6.4	
	95%CI	0.7–2.8	0.8–1.8	0.5–1.4	

Statistical elaboration with one-way ANOVA followed by Fisher's least significant difference (F-LSD)

influenced by both total content of adipose tissue and its distribution (Lee et al. 2013). It is estimated that an increase in waist circumference by 1 cm in children elevates the risk of cardiovascular disorders by 2%.

The outstanding dietary mistakes in Polish adolescents consist of irregular meals, frequent snack consumption, and skipping breakfast, all of which is accompanied by physical inactivity (Wojtyła-Buciora et al. 2015; Kapka-Skrzypczak et al. 2012). The present study showed that obese children aged 7–12 consumed, on average, 1732 kcal/day (108% of RDA). A much lower dietary energy intake of 1480 kcal/day (64% of RDA) was reported by the oldest children investigated aged 16–18. Maier et al. (2011) have reported that the average daily energy consumption among obese children aged 5–8 amounts to 1636 kcal/day, which is grossly in line with that found in the youngest children of the present study above outlined. Other studies show more diverse results. Okręglicka and Bawa (2011) have reported a lower average dietary energy intake in obese children aged 7–13 amounting to 1386 kcal/day. In that study, however, the actual produce consumption seems understated as there is a noticeable lack of snacks, fruit juices, and sweetened beverages listed. On the other side, Falkowska et al. (2011) have reported a considerably average energy intake in overweight children of comparable age amounting to 2232 kcal/day in girls and 2511 kcal/day in boys.

Another dietary mistake often reported in children is excessive fat and sugar consumption (Mahan et al. 2011; WHO 2015). Not only is the consumed fat deposited as adipose tissue, but also excessive carbohydrates and proteins are converted into fatty acids in the liver. The present study showed an excessive consumption of protein and fat, with their dietary intake significantly higher in younger age groups, compared

Table 4 Lipid metabolism in age groups

Variable		7–11 years (n = 27)	12–15 years (n = 25)	16–18 years (n = 32)	ANOVA + F–LSD
					F(2,81) = 2.57; p = 0.082
TC (mg/dL)	Mean ± SD	165.3 ± 32.1	185.3 ± 33.1	168.8 ± 35.9	
	Min–Max	110.0–239.0	124.0–261.0	114.0–284.0	
	95%CI	152.6–178.0	171.6–198.9	155.9–181.8	
					F(2,81) = 1.65; p = 0.198
LDL (mg/dL)	Mean ± SD	100.4 ± 27.1	113.1 ± 30.7	102.1 ± 24.8	
	Min–Max	53.0–168.0	47.0–184.0	61.0–170.0	
	95%CI	89.6–111.1	100.4–125.7	93.1–111.0	
					F(2,81) = 0.70; p = 0.500
HDL (mg/dL)	Mean ± SD	41.9 ± 10.0	39.9 ± 9.1	38.6 ± 12.1	
	Min–Max	22.0–60.0	22.0–62.0	19.0–77.0	
	95%CI	37.9–45.8	36.2–43.7	34.3–43.0	
					F(2,81) = 4.70; p = 0.012
TG (mg/dL)	Mean ± SD	112.4 ± 45.8	160.7 ± 65.9	138.5 ± 57.9	1 vs. 2; p = 0.003
	Min–Max	41.0–211.0	65.0–361.0	65.0–338.0	1 vs. 3; p = 0.083
	95%CI	94.3–130.5	133.5–187.9	117.6–159.4	2 vs. 3; p = 0.148
					F(2,81) = 3.34; p = 0.040
Non-HDL (mg/dL)	Mean ± SD	123.4 ± 31.1	145.4 ± 3.6	130.2 ± 30.4	1 vs. 2; p = 0.013
	Min–Max	61.0–191.0	92.0–215.0	84.0–207.0	1 vs. 3; p = 0.409
	95%CI	11.1–135.7	131.9–158.8	119.2–141.1	2 vs. 3; p = 0.073
					F(2,81) = 2.34; p = 0.103
TC/HDL	Mean ± SD	4.1 ± 1.1	4.8 ± 1.2	4.7 ± 1.2	
	Min–Max	2.2–6.6	2.9–7.1	2.8–7.8	
	95%CI	3.7–4.6	4.3–5.3	4.2–5.0	
					F(2,81) = 3.22; p = 0.045
TG/HDL	Mean ± SD	2.9 ± 1.5	4.4 ± 2.9	3.9 ± 2.1	1 vs. 2; p = 0.016
	Min–Max	0.8–6.6	1.3–16.4	1.4–8.6	1 vs. 3; p = 0.073
	95%CI	2.3–3.5	3.2–5.6	3.2–4.7	2 vs. 3; p = 0.443

Statistical elaboration with one-way ANOVA followed by Fisher's least significant difference (F-LSD)
TC total cholesterol, *LDL* low-density lipoprotein cholesterol, *HDL* high-density lipoprotein cholesterol, *TG* triglycerides

Table 5 Associations between fructose content in the diet and anthropometric indices

Fructose		BMI (kg/m^2)	BMI *z-score*	Waist circumference (cm)	WHtR	%FAT (%)
Total fructose (g)	*r*	0.073	0.070	0.125	0.127	0.146
	p	0.207	0.216	0.090	0.087	0.060
Fructose in vegetables (g)	*r*	−0.037	0.008	−0.077	−0.011	−0.021
	p	0.340	0.463	0.204	0.454	0.411
Fructose in fruits (g)	*r*	0.007	−0.021	0.066	0.020	−0.034
	p	0.468	0.405	0.240	0.416	0.360
Fructose in beverages (g)	*r*	0.115	0.088	0.192	0.174	0.186
	p	0.098	0.161	0.019	0.030	0.023
Fructose in other sources (g)	*r*	−0.089	−0.087	−0.132	−0.120	−0.059
	p	0.160	0.165	0.079	0.100	0.264

r Pearson's coefficient, BMI z-score is according to WHO *WHR* waist-to-height ratio, *%FAT* percent of body fat

Table 6 Associations between fructose consumption and lipid metabolism

Fructose		TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)	Non–HDL (mg/dL)	TC/ HDL	TG/ HDL
Total fructose (g)	*r*	0.122	0.040	−0.056	0.124	0.121	0.093	0.153
	p	0.088	0.331	0.271	0.083	0.093	0.155	0.046
Fructose in vegetables (g)	*r*	0.046	−0.013	0.038	0.083	−0.006	0.057	0.077
	p	0.306	0.442	0.341	0.179	0.472	0.269	0.200
Fructose in fruits (g)	*r*	0.035	0.007	−0.082	−0.026	0.105	0.036	0.018
	p	0.348	0.470	0.184	0.388	0.126	0.348	0.423
Fructose in beverages (g)	*r*	0.141	0.087	−0.066	0.127	0.136	0.070	0.138
	p	0.059	0.172	0.235	0.078	0.068	0.224	0.064
Fructose in other sources (g)	*r*	−0.022	−0.021	0.019	−0.018	−0.009	−0.007	−0.023
	p	0.402	0.412	0.417	0.421	0.459	0.470	0.399

TC total cholesterol, *LDL* low-density lipoprotein cholesterol, *HDL* high-density lipoprotein cholesterol, *TG* triglycerides, *r* Pearson's coefficient

to reference values. Concerning carbohydrates, although their consumption in total did not exceed the recommended reference values for children, there was a notable increase in sucrose consumption. Carbohydrate consumption, on average, was significantly lower at the postpubertal age of 16–18 years (43.4 g/day; 12% of total daily energy intake) than in the remaining younger age groups (13–15 years, 58.9 g/day, 13% of total daily energy intake, and 7–12 years, 66.9 g/day, 16% of total daily energy intake). In a study of Charzewska et al. (2013), sucrose consumption in randomly selected Polish 4-year-olds is estimated at 76 g/day (17% of total daily energy intake). Those authors further report sucrose consumption in the 11–15 age bracket of 84 g/day in boys and 67 g/day in girls (14–15% range of total daily energy intake, respectively).

In the present study, average daily intake of fructose amounted to 19.6 g in children aged 7–12, with the 4.6% percent of energy delivered by it. Fractions of energy delivered by dietary fructose were grossly similar in children also at pubertal and postpubertal age, amounting to 5.9% and 5.2%, respectively. This percentage is relatively low compared with the majority of other studies on the subject. In general, children consume particularly excessive amounts of simple sugars with juices, carbonated and fruit drinks, sweetened tea, energy drinks, and flavored water, which usually is accompanied by reduced intake of healthier complex carbohydrates (USDA 2015b; Wojtyła-Buciora et al. 2015). Maier et al. (2011) have conducted a study among obese German children aged 5–8. The authors report the average fructose consumption in this group of 46 g/day, with about 12% of energy coming from this source. A study conducted in a cohort of about 8 thousand American adolescents aged 12–18 shows an average consumption of fructose of 10.3% (Sun et al. 2011). In a study of Couch et al. (2013), adolescents suffering from type I diabetes, aged 10–22, have fructose consumption estimated at about 35 g/day, corresponding to 8% energy intake. The produce containing added sugar is a significant source of calories. A daily consumption of sweetened beverages providing 150 kcal increases body weight by 6.8 kg annually (Kłosiewicz-Latoszek and Cybulska 2011). Moreover, sweet taste suppresses the feeling of satiety, which increases total food ingestion and consequently body mass, with all medical sequelae (Tappy and Lê 2010; Bray et al. 2004). Maier et al. (2011) have shown that diminishing fructose consumption mitigates BMI increase in children.

It is suggested that excessive fructose intake may impact liver function and the composition of intestinal microflora, leading to metabolic disorders. Excessive fructose consumption has also been associated with a risk of atherogenic changes in the lipoprotein and triglyceride profiles (Jang et al. 2018; Zhang et al. 2017). Reports, such as the one by Sun et al. (2011), showing no real influence of fructose on TG, HDL, glycated hemoglobin, uric acid concentrations, blood pressure,

waist circumference, and BMI are definitely in a minority position.

There are few studies, especially performed in children, concerning the effects of dietary fructose on anthropometric and biochemical indices. A study of Couch et al. (2013) in children with type 1 diabetes confirmed the impact of fructose on the serum TG content, with no appreciable influence on total cholesterol, LDL and HDL lipoproteins, or blood pressure. The authors report that an increase in fructose intake by 22 g, contained in approx. 340 ml of alcohol-free beverages, is associated with TG increase by 4%. Pollock et al. (2012) have conducted a study in a group of children, aged 14–18, and have demonstrated that serum TG and LDL content and visceral adipose tissue mass increased and HDL content decreased, with an increase in fructose intake. The authors indicate that a greater fructose intake in adolescents associates with numerous markers of cardio–metabolic risks. They emphasize that these associations are indirectly related to visceral obesity. The findings of the present study are in line with those above outlined, showing an association between fructose intake and waist circumference, WHR, % FAT, TC, TG, non-HDL, and TG/HDL, especially when sweetened drinks were the source of this carbohydrate. Overall, the available literature points to the role of individual nutrients, with a particular emphasis on limiting the intake of simple sugars, and dietary patterns and habits, including the composition and frequency of meals, as significant factors in obesity prevention (Kapka-Skrzypczak et al. 2012; AHA 2011; Lanfer et al. 2010).

In conclusion, we believe we have shown that dietary habits constitute one of the main factors influencing the development of excessive body weight and obesity. Fructose appears a particularly unbeneficial component of a diet to this end. This study demonstrates an undesirable enhancing influence of fructose on adipose tissue and its distribution within the waist and also on the atherogenic lipid profile. Metabolic diet-related disorders may result not only from the amount of fructose consumed but also from inadequate proportions of other macronutrients.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by the Bioethics Committee of Warsaw Medical University in Poland.

Informed Consent Informed consent was obtained from all individual participants included in the study and/or their parents/legal guardians.

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Vaccination Against Measles, Mumps, and Rubella in the Light of Current Epidemic Threats: Unjustified Postponement

Hanna Czajka, Szymon Czajka, Katarzyna Anna Dyląg, Elżbieta Borek, and Ernest Kuchar

Abstract

A worrying increase in the number of measles cases has been noted recently in Poland, which may have to do with a decreasing proportion of children vaccinated against measles, mumps, and rubella (MMR) in the second year of life (<95%). For many years, MMR vaccination in children has been associated with a fear of allergy to eggs. This study seeks to define the reason and justification for postponing MMR vaccination in a population of children referred to the outpatient specialist immunization clinic. One hundred and thirty eight (138) children, mean 24.5 ± 26.6 months, with a history of past allergies, in whom the first-time MMR vaccination was delayed by family doctors for fear of allergic reactions, were enrolled into the study. The mean delay in a vaccine shot was 12.3 ± 26.9 months. There were 101 children who displayed a distinct allergy to the egg proteins, among other accompanying types of allergy. All of the 138 children were found eligible to receive MMR vaccine at the visit to the clinic. No early allergic responses were noticed in any of the children. There were negligible delayed allergic responses in six children, all from the egg allergy group. We conclude that MMR vaccination in children with egg allergy is safe and can be conducted on the outpatient basis without any specific precautions or safety measures. Delays in vaccination were unjustified and may jeopardize children's health. There is a need for insightful education of primary care doctors concerning of MMR vaccination safety, particularly when allergy is suspected, to avoid unduly and potentially harmful delays.

H. Czajka
Faculty of Medicine, Rzeszow University, Rzeszow, Poland

St. Louis Regional Specialized Children's Hospital, Cracow, Poland

S. Czajka (✉)
School of Medicine, Medical University of Silesia, Katowice, Poland
e-mail: szymon_czajka@wp.pl

K. A. Dyląg
Medical College of the Jagiellonian University, Cracow, Poland

St. Louis Regional Specialized Children's Hospital, Cracow, Poland

E. Borek
St. Louis Regional Specialized Children's Hospital, Cracow, Poland

E. Kuchar
Department of Pediatrics with Clinical Assessment Unit, Warsaw Medical University, Warsaw, Poland

Keywords
Allergy · Egg proteins · Immunization · Measles · MMR vaccine · Vaccination · Vaccine coverage rate

1 Introduction

According to the epidemiological data published by the European Center for Disease Prevention and Control Publications (ECDC) in the last few years, the number of cases of measles increased significantly in the EU countries. Between September 1, 2017, and August 31, 2018, 13,547 cases of measles have been reported, of which 9,364 (69%) were laboratory-confirmed. Measles has been reported in Romania (5088), France (2702), Greece (2289), and Italy (2248). There were 33 measles-related deaths between January and October 2018. Out of the 12,162 measles cases with the known children's age, 82% were unvaccinated and 11% were immunized with one dose of measles-containing vaccine (ECDC 2018a). Moreover, there have been 32,489 cases of measles in Ukraine in 2018, including 19,476 in children, reported till October 9, with 14 deaths (ECDC 2018b).

Vaccination against measles carried out for decades has led to a significant decrease in the appearance of the disease in countries which implemented universal prophylaxis. In Poland, a mandatory vaccination against measles for children aged 13–15 months was introduced in 1975. The percentage of vaccinated children has since then been steadily increasing reaching 79% in 1988, which was accompanied by gradually decreasing morbidity. However, there were two large epidemics reported in 1984 and 1990, with morbidity at a level of 148/100,000. The majority of cases were diagnosed in adolescents; hence, a booster of measles vaccination was introduced in the seventh year of life to avoid further epidemics. After the introduction of a combined measles, mumps, and rubella (MMR) in 2004, which substituted for the monovalent measles vaccine, the booster was postponed to the tenth year of age.

According to the data published by the National Institute of Public Health-National Institute of Hygiene in Warsaw, the number of cases of measles in Poland is subject to periodic fluctuations. For instance, there were 166 cases of measles reported in Poland in 2016 (morbidity of 0.35/100,000), whereas only 48 cases were observed in 2015 (morbidity of 0.12/100,000). In 2017, the number of patients diagnosed with measles amounted to 63, but between January and the end of October, 2018, there were 144 cases of measles noted. The risk of measles sharply increases in the population when the percentage of vaccinated patients decreases below 95%. Thus, increasing the number of people evading vaccinations increases the incidence of measles in such countries as Romania, France, Italy, or Great Britain, where the vaccine coverage rate has been decreasing in the last few years (ECDC 2018c). The same tendency is recently observed in Poland. The December 2017 data show that the percentage of children, born in 2015, vaccinated against measles has been about 94%, with some provinces, for instance, the Mazovian province dropping to the alarming level of below 90% (PZH 2018). In this situation, the second dose of MMR has been shifted from 10 to 6 years of age in the Polish vaccination program for 2019.

On the other side, an increase in allergic diseases has been observed worldwide (Kelso 2014; Ainsworth et al. 2010; Patja et al. 2001; James et al. 1995). Parents of children allergic to egg proteins are wary of allergic reactions after MMR vaccine administration. Such concerns seem unjustified as observational studies conducted for many years demonstrate a lack of allergic responses to the dram of egg proteins contain in MMR vaccine (Dreskin et al. 2016). It is most unfortunate that the parents' concerns are shared by some physicians caring for children who unreservedly refuse to qualify children for vaccination if they are allergic to egg proteins or even to whatever different allergen. Patients are often referred to specialist immunization clinics or hospital vaccination centers, which delays vaccination. In view of the epidemiological situation outlined above, the present study seeks to define the reasons for postponing MMR

vaccination and to determine to what extent doctors' decisions to refer the children with a suspicion of allergy to egg proteins to a specialist immunization clinic were justified.

2 Methods

2.1 Study Setting and Patients

This study was performed in an outpatient immunization clinic of St. Louis Regional Specialized Children's Hospital in Krakow, Poland. The clinic consults an average of 5600 patients annually, of whom 1150 come for an initial consultation. Most of the children are from high-risk groups (preterm infants, oncologic patients, allergies, neurologic, cardiologic, and other chronic comorbidities). The files of 184 children, who visited the clinic between January 2013 and September 2016, were reviewed. They had the ICD diagnosis of hen egg allergy and were referred to the clinic due to the need of MMR vaccination. Twenty five (14%) children did not meet the inclusion criteria due to the presence of referral mistakes, incomplete data, or MMR vaccination already performed in a GP's office. Out of the remaining 159 files, 138 children (84 boys and 54 girls) who had not been earlier vaccinated with the first dose of MMR vaccine were included in the final analysis (Fig. 1). The mean age of the 138 children was 24.5 ± 26.6 months, with the oldest patient of 15 years and the youngest one of 12 months of age. Ninety three (67.4%) children lived in urban areas and 45 (32.6%) lived in a rural area. The mean age at which the allergy symptoms first appeared was 5.6 ± 9.9 months.

Children were selected from a large group of ICD referrals, such as L20, atopic dermatitis; L27, dermatitis due to substances taken internally; T78.1, other adverse food reactions; not elsewhere classified T78.2, anaphylactic shock; unspecified, T78.4, allergy; unspecified, J45, asthma; and L50, urticaria. The children who were referred to the clinic in order to receive delayed MMR doses were the only ones included in the analysis. The parameters considered were age, gender, place of residence, history of allergy, age at allergy diagnosis, severity of allergy symptoms, diet, and delay of MMR vaccination in relation to the official vaccination schedule.

2.2 Study Procedure

MMR vaccine was administered during the first visit of a child to the outpatient immunization clinic or at the earliest convenient time shortly afterward. Different postvaccination observation periods were recommended, depending on the severity of allergic symptoms. The standard observation lasted for 30 min. It was extended up to 3 or more hours as required. We assessed the injection site reactions, fever, and the general patient's condition. To ensure a full knowledge on the immunization safety, we conducted a follow-up telephone interview among parents of vaccinated children 30 days after vaccination. The following question was asked: How did the child feel in the first 3–4 days and later up to the 15th day after vaccination? The interrogation concerned fever, redness of injection site, generalized rash, exacerbation of any allergy, swollen lymph nodes, or the presence of any other new or disturbing symptoms. The vaccines used were Priorix (GlaxoSmithKline Biologicals s.a.; Rixensart, Belgium) and MMRVAXPRO (Merck Sharp & Dohme Limited; Hoddesdon, Hertfordshire, UK).

2.3 Statistical Analysis

Continuous variables were expressed as medians, or means ±SD categorical variables were expressed as counts and percentages. Student's *t*-test or Mann Whitney U test was used for continuous data and a chi-squared test or Fisher's exact test for qualitative data. We used a logistic regression analysis to identify risk factors for a delay in MMR vaccination. A p-value <0.05 defined statistically significant changes. Data were processed using a commercial Statistica v12.5 package (StatSoft, Inc., Tulsa, OK).

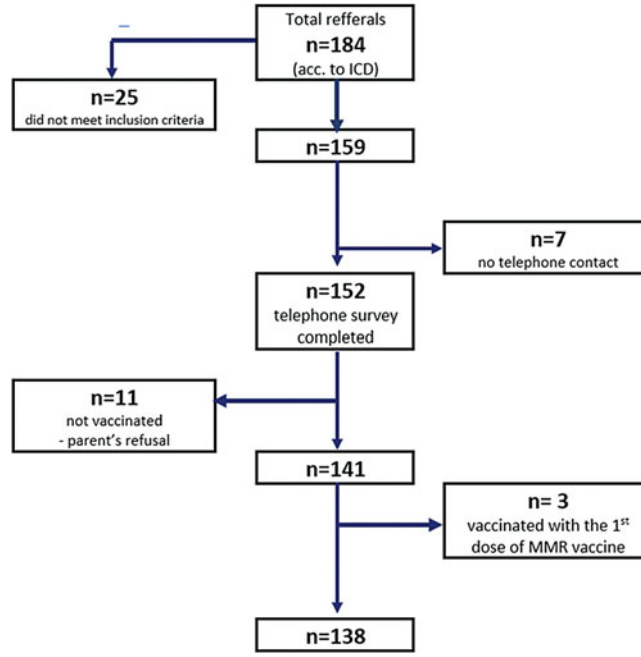

Fig. 1 Flow diagram of the patient recruitment procedure

3 Results

The final study population of 138 children was divided into two groups: children who among various allergies had a distinctly positive history of allergy to egg proteins ($n = 101$; 73.2%) and children with various allergies exclusive of allergy to egg proteins ($n = 37$; 26.8%). The severity of allergic reactions was assessed during history taking. Allergy manifestations to egg proteins in the 101 children were stratified into: mild (rash/dermatitis, gastrointestinal manifestations, and pollenosis) in 67 (66.3%) children, moderate (urticaria and swelling) in 29 (28.7%) children, and severe reactions (anaphylaxis) in 5 (4.0%) children. Thus, the present referral to the specialist immunization clinic could have been justified in case of the latter 34 patients with more severe allergy manifestation, although the current recommendations do not point to the absolute requirement of hospitalization. There were no patients with a history of allergy to neomycin or gelatin. There were no appreciable differences between the groups concerning age at initial visit, gender, gestational age, and place of residence (Table 1).

MMR vaccine was administered to all 138 children. None of the children showed any signs of an early allergic reaction. Delayed reactions, up to 72 h after vaccination, verified during a telephone survey, appeared in 6 (5.9%) children who all came from the 101 children group with allergy to egg proteins. The allergy manifestations included common cold symptoms and diarrhea in two children each and atopic dermatitis and lip swelling in one child each. None of these symptoms were severe and none required hospitalization. A cause–effect relationship between vaccination and atopic dermatitis and lip swelling appeared highly likely. However, such relationship was dubious in case of vaccination and the occurrence of diarrhea and cold-like infection. A significant relationship was noticed between the presence of redness and swelling where the shot was given and the delay in vaccine administration (Table 2).

Considering the whole study population of 138 children, vaccine was administered according to the currently recommended vaccination schedule, i.e., the first shot between the 13th and 14th month of life, in 21 (15.2%) children, whereas in 117 (84.8%) children vaccination was delayed for

Table 1 Demographic characteristics of the patients studied

Variable	All children $n = 138$	Children with allergy to egg proteins $n = 101$ (73.2%)	Children without allergy to egg proteins $n = 37$ (26.8%)
Age (months)	24.6 ± 26.6	28.4 ± 33.3	21.6 ± 18.2
Gender male; *n* (%)	84 (60.9)	62 (61.4)	22 (59.5)
Gender female; *n* (%)	54 (39.1)	39 (38.6)	15 (40.5)
Preterm born; %	5 (3.6)	3 (3.0)	2 (5.4)
Living in a city; *n* (%)	93 (67.4)	65 (64.4)	28 (75.7)
Living in a rural area; *n* (%)	45 (32.6)	36 (35.6)	9 (24.3)

Data are means ±SD or number and percentage of patients, *n* (%)

Table 2 Data concerning allergy. Summary of regression analysis, with the vaccination delay at initial visit (days) as a dependent variable

Variable	B ± SE	p-value
Allergic reaction after eating an egg yes/no	−1.30 ± 3.80	0.733
Rash yes/no	−5.74 ± 4.63	0.217
Urticaria yes/no	4.35 ± 4.41	0.326
Swelling at injection site yes/no	13.59 ± 4.33	0.002
Gastrointestinal manifestations yes/no	0.13 ± 0.18	0.474

B unstandardized regression weight

about a year. The mean vaccination delay amounted to 12.3 ± 26.9 months. Out of the 37 children with no report of egg allergy, only 4 (10.8%) were vaccinated according to the schedule, and the mean delay in vaccination amounted to 20.5 ± 40.8 months. Out of the 101 children with the egg allergy reported, 17 (16.8%) were vaccinated according to the schedule, and the mean delay in vaccination amounted to 9.3 ± 19.0 months. Differences in the number of vaccinated children and in the delay in the scheduled vaccine administration were insignificant between the groups with and without egg allergy.

4 Discussion

Recommendations concerning allergies in children and the safety of MMR have evolved during the last two decades from the inhospital to outhospital treatment, along with the evanescing content of egg proteins in the vaccine (Dreskin et al. 2016; Franceschini et al. 2015; Andersen and Jørgensen 2013; Khakoo and Lack 2000). In the 1980s and 1990s, it was recommended to take caution while administrating MMR vaccine to children who had had a positive history of allergy to egg whites and to conduct skin tests before vaccination (Baxter 1996; Herman et al. 1983). Other studies, however, questioned the necessity of performing skin tests (Aickin et al. 1994; Kemp et al. 1990). As early as in 1971, it was shown that the ovalbumin protein was undetectable in a measles vaccine when assessed with the measurement accuracy of microgram *per* milliliter (O'Brien et al. 1971). Contemporary studies conclusively demonstrate that the protein of egg whites in the MMR vaccine is but at a trace concentration or just unmeasurable. Thus, the vaccine may be safely and restriction free administered to patients with allergy to this protein (Kelso 2014). A British study, reviewing the 1996–2009 literature and the pediatricians and family doctors adherence to vaccination administration, has shown that 81% of children in whom vaccination is delayed for more than 30 days do not actually meet criteria for severe allergic reactions to egg proteins, which obviates the need to vaccinate them in the hospital setting (Ainsworth et al. 2010). It is our experience that physicians are not savvy enough about the

composition of MMR vaccine and its use in allergic children. In the current study, allergy to egg proteins was unconfirmed in 37 (26.8%) children of those referred to the immunization clinic and MMR vaccine caused no allergic reactions. In the 101 children, who had a positive history of egg allergy, vaccination failed to cause any acute allergic reactions as well. It resulted in just six delayed, mild, and fleeting reactions that neither required hospitalizations nor raised the parental concern.

In this study, we found that 84.8% of children referred to the immunization clinic had a delay in the implementation of MMR vaccination program of the mean duration of 12.3 months. This delay pointedly demonstrates a need to expedite the vaccination procedure, with a careful consideration for the child's safety and comfort. Numerous studies have shown that MMR vaccination can be safely performed in a GP's office (Franceschini et al. 2015; Andersen and Jørgensen 2013; Cronin et al. 2012; Patja et al. 2001; James et al. 1995). British authors suggest that patients inappropriately referred to hospital should nevertheless be vaccinated on the day of their visit, rather than referred back to the primary care doctor (Ainsworth et al. 2010). In contradistinction, a New Zealand study suggests that children without indications for inhospital MMR vaccination should be referred back to the family doctor's office (Goodyear-Smith et al. 2005). We share the British opinion that it is better to perform an already delayed vaccination at the first opportunity instead of referring the patient back to the doctor's office causing further undue delay in the implementation of the vaccination program. A final decision hinges, however, on the attitude of child guardians who often express less concern about adverse effects in the hospital setting, with an extended hours-long observation time.

Reviewing the factors that had a particular effect on the postponing of a currently scheduled MMR vaccination, we found that a major impact had redness and swelling where the shot was given. Such a reaction worries parents, and strangely enough also doctors, which delays similar interventions in the future. A positive assessment of MMR vaccine safety in children with allergies found in the present study is in line with the opinions of other medical centers which encourage a full implementation of the vaccination recommendations (Dreskin et al. 2016). The question remains whether pediatricians and family doctors are savvy of these recommendations and would fully stick to them, which unfortunately does not seem convincingly certain in the local medical community.

In conclusion, MMR vaccination in children with allergy, including egg allergy, is safe and can be conducted without additional consultations or safety measures. Children with egg allergy inappropriately referred to the specialist outpatient immunization clinic or hospital should be vaccinated on the day of their visit, rather than referred back to the primary care physician. There seems a strong need for insightful education of primary care doctors in the realm of vaccination safety, particularly in case of suspected allergic reactions. It would be worthwhile to create a unifying Polish standard for vaccinating children with allergic diseases, with emphasis on measles, mumps, and rubella (MMR) vaccinations.

Conflicts of Interest The authors declare no conflicts of interest concerning this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. A local Ethics Committee approved the study protocol.

Informed Consent The need to obtain the consent from individual participants or their guardians to be included in the study was waived by the Ethics Committee due to a retrospective nature of the study consisting of reviewing the medical history files only.

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Variability of Dry Eye Disease Following Removal of Lacrimal Glands in Rats

Janusz Skrzypecki, Huc Tomasz, and Ciepiaszuk Karolina

Abstract

Removal of lacrimal glands is used as a viable model of dry eye disease in rats. However, there is no uniform agreement on the disease severity following different variants of the procedure. The interpretation of the modeled dry eye disease also is biased by the interchangeable use of male and female rats. Therefore, this study seeks to define the features of dry eye disease following removal of the extraorbital lacrimal gland, with or without excision of the infraorbital lacrimal gland in male and female rats. The experiments were performed in 12-week-old female and male Sprague-Dawley rats. The baseline blink rate and fluorescein score were assessed. Subsequently, rats underwent isolated removal of the extraorbital gland, removal of the extraorbital gland combined with excision of the infraorbital gland, or a sham surgical procedure. The assessment of blink rate and fluorescein scores was repeated 28 days following surgery. Corneas were collected for histological analysis. We found that the blink rate and fluorescein score increased in all of the experimental groups, except the control group and the male rats that underwent isolated removal of the extraorbital lacrimal gland. Histopathological analysis revealed the thinning and edema of the epithelium in all groups, except the control group. These changes were most pronounced in female rats following combined removal of extraorbital and infraorbital lacrimal glands. In conclusion, severity of dry eye disease in the rat model is influenced by both gender and the extent of surgical removal of lacrimal glands. Combined excision of lacrimal glands in female rats produced the most severe pathological changes, whereas isolated excision of the extraorbital lacrimal gland in male rats led to the least severe changes.

Keywords

Dry disease · Eye · Animal model · Cornea · eye · Lacrimal glands · Tear film

1 Introduction

Dry eye disease is a chronic condition caused by abnormalities of the tear film. Decreased production or increased evaporation compromises the ocular surface, leading to desiccation of epithelial cells and their apoptosis. That, in turn, triggers inflammation, which perpetuates a vicious circle of the disease (Perry 2008). The estimated prevalence of dry eye disease ranges from 5% to 50% (Stapleton et al. 2017). Although mild forms of

J. Skrzypecki (✉), H. Tomasz, and C. Karolina
Department of Experimental Physiology and Pathophysiology, Laboratory of Center for Preclinical Research, Warsaw Medical University, Warsaw, Poland
e-mail: jskrzypecki@wum.edu.pl

the disease are often disregarded by patients and doctors, severe dry eye disease, due to scarring or secondary infections, is a potentially blinding condition (Deswal et al. 2017).

Considering the burden of dry eye disease and its complex pathophysiology, many experimental studies including in vitro and animal models have been developed to broaden our understanding of the disease and to evaluate potential treatments (Higuchi et al. 2011; Barabino et al. 2004; Offord et al. 1999). Although cell lines are viable for determining the safety of treatment and its anti-inflammatory properties, no clinically relevant data, i.e., a blink rate or fluorescein staining score, has by far been derived from those studies. Given a complex environment of the ocular surface, animal models are preferable to in vitro models in the studies on the pathophysiology and treatment of dry eye disease. In brief, animal models of dry eye disease emulate increased evaporation or decreased secretion of the tear film (Schrader et al. 2008). The former is achieved by cauterization of Meibomian gland orifices or genetic modification of Meibomian gland function (Ehrmann and Schneider 2016; Gilbard et al. 1989), whereas the latter by removal of lacrimal glands (Barabino and Dana 2004). Rats are equipped with the main lacrimal gland (exorbital lacrimal gland), accessory lacrimal gland (infraorbital lacrimal gland), and Harderian lacrimal glands (intraorbital lacrimal gland) (Percy et al. 1989). Given the lack of technical difficulties related to the procedure, removal of the main lacrimal gland is one of the most frequently utilized models of tear-deficient dry eye disease in rats (Fujihara et al. 2001). Although studies on tear-deficient models are popular and efficient, they might be difficult to interpret. Firstly, significant differences in the disease severity have been reported following excision of the extraorbital lacrimal glands in rats (Higuchi et al. 2010). Secondly, some studies are performed following isolated removal of the extraorbital gland and some following combined extraorbital and infraorbital lacrimal gland excision. Finally, studies, which have been published so far, utilize male and female animals interchangeably (Meng et al. 2015; Stevenson et al. 2014). Considering the influence of sex hormones on the remaining lacrimal tissue and on the Meibomian glands, comparisons between the experiments may be skewed. Therefore, the present study seeks to define the benchmark characteristics of dry eye disease in female and male rats following removal of main lacrimal gland, with or without excision of accessory lacrimal glands.

2 Methods

The experiments were performed in 12-week-old male ($n = 24$) and female ($n = 24$) Sprague-Dawley rats. Throughout the study, animals stayed in their housing cages with access to water and chow ad libitum. Air temperature was set at 23.8 °C and humidity at 54.4%. All female rats were put in one room at birth, which enabled the synchronization of menstrual cycles and similar exposure to female sex hormones.

2.1 Experimental Protocol

On Day 1, baseline blink rate (BR) and fluorescein score (FS) were assessed in all animals. Subsequently, rats underwent surgical removal of the extraorbital lacrimal gland (main lacrimal gland) ($n = 16$; female = 8, male = 8), removal of the extraorbital gland with removal of the infraorbital lacrimal gland (accessory lacrimal gland) ($n = 16$; female = 8, male = 8), or sham surgery ($n = 16$, female = 8, male = 8). On Day 28, BR and FS were reassessed.

2.2 Surgery

All procedures were performed unilaterally and were as follows.

Isolated Removal of the Extraorbital Lacrimal Gland The skin was incised from the lateral canthus of the eye toward the auricle. The connective tissue was dissected, and the extraorbital lacrimal gland was visualized just anterior to the auricle. All relevant vessels were closed with 4-0 nylon

sutures. Subsequently, the extraorbital lacrimal gland was released from the connective tissue and removed. The skin was closed with 4-0 nylon sutures.

Combined Removal of the Extraorbital Lacrimal and Infraorbital Lacrimal Glands Following removal of the extraorbital lacrimal gland, the superficial temporal artery was visualized and dissected rostrally. Infraorbital lacrimal gland, which lies over the temporal artery and posterior to the lateral canthus, was removed. The skin was closed with 4-0 nylon sutures.

Sham Surgery The skin was incised and connective tissue dissected as described above. The extraorbital lacrimal gland and the infraorbital lacrimal gland were visualized. No lacrimal tissue was removed. The skin was closed with 4-0 nylon sutures.

2.3 Grading Scores

Fluorescein Score (FS) was measured under short anesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg, i.p.). One drop of fluorescein was instilled into both eyes and rinsed with 1 ml of 0.9% NaCl 2 min later. Photographs of the corneas were taken under cobalt blue light and 14x magnification. Subsequently, FS (range, 1–5) was assessed by two blinded observers, and it was averaged for the final result.

Blink Rate (BR) was measured by two independent observers for 2 min. During the count, rats were housed in their living cages. To minimize a possible error, the cover of the cage was removed. Two counts were averaged for the final result. BR was expressed as the number of blinks per minute.

2.4 Histopathology

Tissues sections were fixed in 10% buffered formalin and embedded in paraffin wax. Sections of 3–4 μm thick were stained with hematoxylin and eosin. A general histopathological examination was performed under the magnifications of 10x, 40x, and 100x (objective lens) and 10x (eyepiece). Photographic documentation was performed. Additionally, the following morphometric measurements were made: width of the cornea, height of the anterior epithelium of cornea, and number of cell layers in the epithelium at magnification of 40x (objective lens). The ratio of the height of the epithelium to the number of cell layers was calculated. All histological procedures were performed using a standard light microscope Olympus BX41 and cellSens software (Olympus Corporation, Tokyo, Japan).

2.5 Statistical Analysis

Normality of data distribution was tested with the Kolmogorov-Smirnov test. Continuous variables and discrete variables were expressed as means ±SE and as medians ±median absolute deviation (MAD), respectively. Normally distributed data were compared with Student's t-test, one-way analysis of variance (ANOVA), or one-way analysis of variance for repeated measurements (RM-ANOVA). Discrete variables were analyzed with the Mann-Whitney U and Kruskal-Wallis tests, whereas categorical data with chi-squared test. Significance was set at the level of $p<0.05$.

3 Results

BR and FS were similar between the groups at the beginning of the experiment. Both increased significantly in all groups 1 month after surgical procedures ($p<0.05$), except sham controls and the male rats that underwent isolated removal of the extraorbital lacrimal gland. The highest BR and FS were observed in female rats following the combined extraorbital and infraorbital lacrimal gland excision ($p<0.05$). BR and FS were lower, to a similar extent, in female rats after the extraorbital lacrimal gland excision and in male rats after the combined procedure ($p<0.05$) (Figs. 1 and 2).

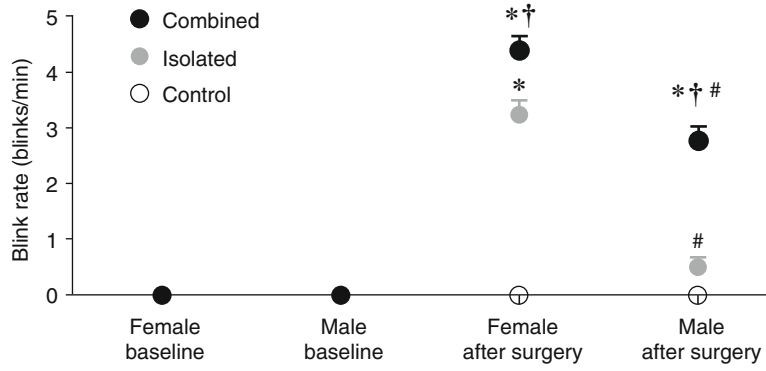

Fig. 1 Blink rate, expressed as means ±SE. *Combined* removal of both extraorbital and infraorbital lacrimal gland, *isolated* removal of extraorbital lacrimal gland alone, *control* sham procedure; *$p<0.05$ for 30 days after surgery vs. baseline; †$p<0.05$ combined vs. isolated vs. control; #$p<0.05$ male vs. female

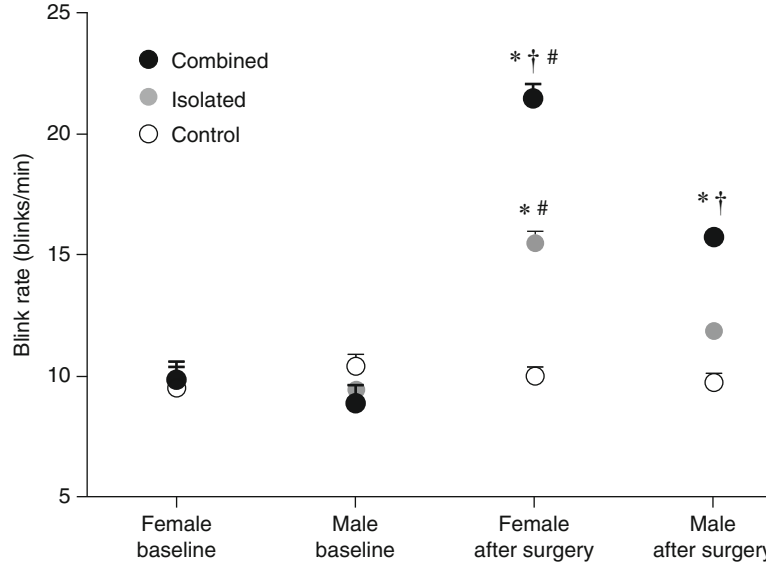

Fig. 2 Fluorescein score, expressed as medians ±MAD. *Combined* removal of both extraorbital and infraorbital lacrimal glands, *isolated* removal of extraorbital lacrimal gland alone, *control* sham procedure; *$p<0.05$ for 30 days after surgery vs. baseline; †$p<0.05$ combined vs. isolated; #$p<0.05$ male vs. female

3.1 Histopathology

In all of the studied groups, the number of epithelial layers was lower than that in sham controls 1 month after surgery ($p<0.05$). The lowest number was found in female rats which underwent the combined extraorbital and infraorbital lacrimal gland excision ($p<0.05$). That was followed by female rats following the isolated extraorbital lacrimal gland removal and male rats after the combined extraorbital and infraorbital lacrimal gland removal ($p<0.05$). The least significant changes were noted in male rats after the isolated extraorbital lacrimal gland excision. The A/B index, i.e., ratio of epithelial thickness to the number of epithelial layers, followed a reverse pattern among the studied groups (Table 1) (Fig. 3).

4 Discussion

The major finding of this study was that combined and isolated excisions of the extraorbital lacrimal gland in female and male rats produced

Table 1 Histological analysis of corneal parameters

	F – combined	M – combined	F – isolated	M – isolated	F – control	M – control
A – Cornea thickness (μm)	165.3 ± 8.0	170.6 ± 5.0	170.6 ± 6.3	176.2 ± 6.7	173.5 ± 7.1	173.5 ± 6.0
B – Epithelium thickness (μm)	33.0 ± 1.9	37.6 ± 1.6	30.1 ± 2.3	34.1 ± 1.5	36.4 ± 2.4	36.4 ± 3.5
No. epithelium layers	2.7 ± 0.1$^{*\#\dagger}$	3.7 ± 0.3*	3.4 ± 0.3*†	4.8 ± 0.3	5.1 ± 0.3	5.1 ± 0.2
A/B index	12.3 ± 0.5$^{*\#\dagger}$	10.4 ± 0.6*	9.1 ± 0.7*†	7.1 ± 0.3	7.1 ± 0.2	7.2 ± 0.2

A, B, and A/B were expressed as means ±SE; number of epithelium layers was expressed as median ± MAD
M male, *F* female, *combined* removal of both extraorbital and infraorbital lacrimal glands, *isolated* removal of extraorbital lacrimal gland alone, *control* sham surgery
*p<0.05 vs. control; $^{\#}p$<0.05 male vs. female; †<p0.05 combined vs. isolated

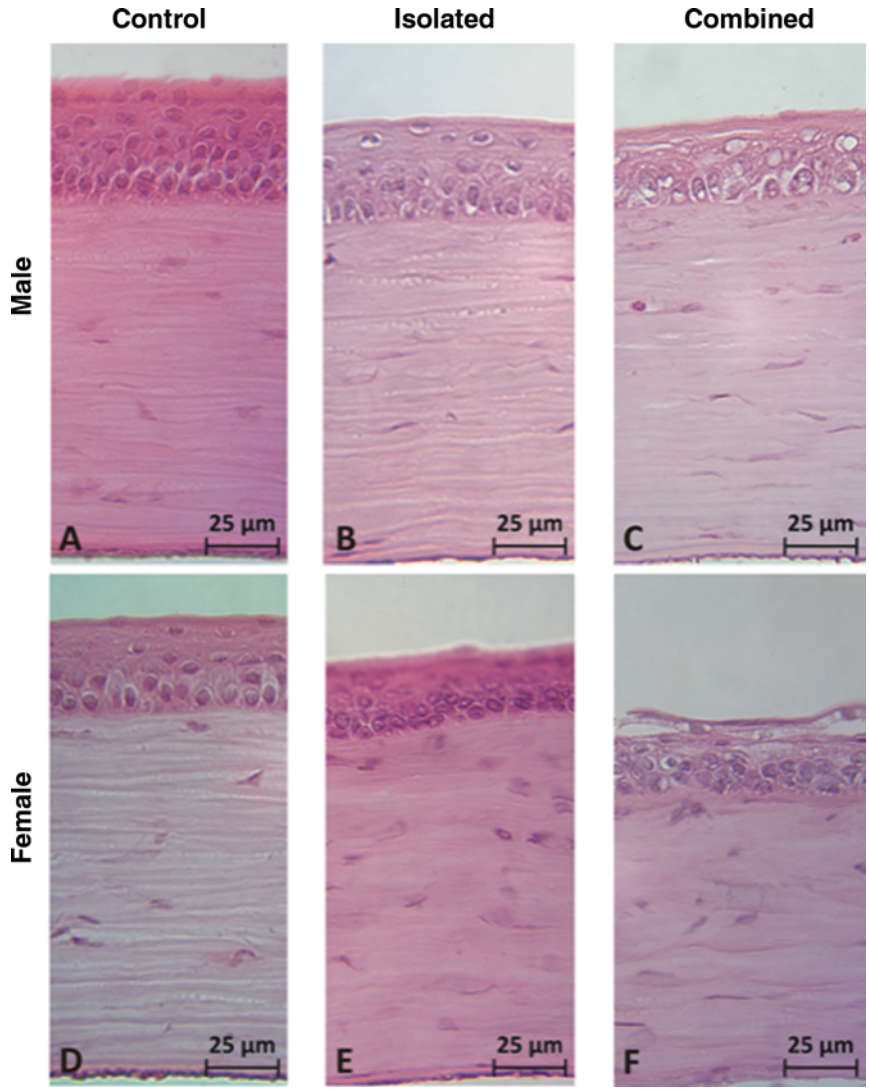

Fig. 3 Histopathological picture of the epithelium layers in the cornea. A, B, C – male rats; D, E, F – female rats; *Combined* removal of both extraorbital and infraorbital lacrimal glands, *Isolated* removal of extraorbital lacrimal gland alone, *Control* sham procedure

a model dry eye disease of diversified severity. Combined excision of the extraorbital and infraorbital lacrimal glands in female rats led to the most severe form of the disease. Less severe changes were induced by isolated extraorbital lacrimal gland removal in female rats and by combined excision in male rats. The least severe changes were observed following isolated extraorbital lacrimal gland excision in male rats.

Several symptomatic discrepancies in the models of dry eye disease have previously been reported. Some studies have shown that clinically significant symptoms, i.e., increased BR and FS, are manifest after isolated excision of the extraorbital lacrimal gland (Higuchi et al. 2010; Fujihara et al. 2001). On the other hand, Meng et al. (2015) have shown that only subtle symptoms may be elicited by such excision, and it takes a combined gland removal to obtain more pronounced symptoms in male Sprague-Dawley rats. In line with the study above outlined, in the current study we found that although isolated removal of the extraorbital lacrimal gland produced only subclinical symptoms of dry eye disease, a combined procedure led to clinically significant symptoms in male rats. However, both procedures produced significant symptoms in female rats. Likewise, histopathology revealed more significant changes in female rats. It has been previously shown that female gender is related to a greater prevalence of dry eye disease. Although various hypotheses have been coined to explain this observation, e.g., increased prevalence of Sjogren disease in females or the effect of estrogens on lacrimal and Meibomian glands, no uniform theory has been established (Sullivan et al. 2017). We believe that a greater severity of dry eye disease in female rats could be attributed to the effect of estrogens on the lacrimal tissue, remaining after surgery, conjunctival goblet cells, or meibomian glands.

The current study is subject to some limitations. It cannot be excluded that a longer than the 4-week-long observation period employed in this study after isolated removal of extraorbital lacrimal glands would reveal more significant symptoms in male rats. Other authors, however, have reported that symptoms of dry eye disease are noticeable as soon as a week following removal of lacrimal glands (Meng et al. 2015). Despite the limitations we believe we have convincingly demonstrated that combined and isolated removal of the extraorbital lacrimal glands in female and male rats might be utilized as a reproducible model of dry eye disease, with the provision of gender-related differences in disease severity.

Conflicts of Interest The authors report no conflicts of interest in relation to this article.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The study was approved by a local Bioethics Committee.

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