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EDITORIAL

From Crisis to Collaboration: Addressing the Neurologist Shortage in the Caribbean and Beyond

A Ali[1] and P Carlen[2]

The global shortage of neurologists is no longer a silent crisis—it is a rapidly worsening threat to public health, particularly in low- and middle-income countries (LMICs) and underserved regions of high-income countries (HICs) such as Canada. Nowhere is this more evident than in the Caribbean, where access to specialist neurological care remains chronically inadequate. In Jamaica, a country of nearly three million people, only six full-time neurologists are available, mirroring the stark neurologist-to-population ratios seen across the Global South (1).

This situation is not merely a matter of numbers. It reflects deeper structural blind spots: neurology's persistent under-representation in national health plans, a lack of coordinated training and retention strategies, and an over-reliance on foreign donations, in the form of temporary Cuban personnel, or temporary solutions that ignore local realities. These problems are compounded by 'neurophobia' in undergraduate education (2), digital transitions that sometimes sideline rather than support clinicians (3), and international initiatives that too often exclude the voices of those working on the ground, possibly representing implicit bias (4, 5).

Neurophobia, a term used to describe the fear and misunderstanding of neurology among medical students and general practitioners, remains a critical barrier to neurological workforce expansion (2). It begins early in medical education, driven by poor neurology teaching, limited exposure to patients with neurological disorders, and the perception that neurological conditions are complex and offer little scope for effective intervention. This negative perception continues into post-graduate training, deterring new entrants and contributing to attrition. Curricular reform that includes more clinically accessible teaching, early exposure to role models, and integration of neurology with other medical disciplines is essential. National and institutional efforts to address neurophobia through continuing medical education and targeted mentorship programmes could help demystify the specialty and potentially attract more students into the field.

Compounding this challenge is the rising tide of burnout among neurologists globally (6, 7). In LMICs, burnout is exacerbated by inadequate staffing, a lack of diagnostic tools, overwhelming patient volumes, and professional isolation. The emotional burden of caring for chronically ill patients with few treatment options adds further strain. Without supportive work environments and access to professional development, neurologists often feel trapped and undervalued. In the Caribbean and other LMICs, this leads not only to early retirement but also to migration. Recognising burnout as a systemic issue rather than a personal failing is crucial. Investments in peer support networks, sabbaticals and flexible career pathways are practical interventions. HICs must also prioritise workforce well-being, especially in rural and remote communities where service demands are high and resources scarce (7).

While Canada has greater absolute numbers of neurologists, it too faces inequities especially in remote, rural and Indigenous communities (8). These challenges, when juxtaposed with those in the Caribbean, highlight an opportunity: greater strategic cooperation between countries with shared histories, similar population health profiles, and, potentially, complementary strengths.

Despite many attempts by the first author over the years, mentees who have left for neurological training only infrequently return, opting to benefit from greater opportunities—academic and financial—in the places they have trained. As an example, a Canada–Caribbean partnership, built around workforce training, shared digital platforms, cross-border mentorship, and twinned centres of excellence, could meaningfully address these chronic shortages. Canadian neurology departments

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could support supervised rotations and remote Continuing Medical Education for Caribbean trainees. Meanwhile, suitably trained and licensed Caribbean-based neurologists could reciprocally help extend care into Canada's underserved diaspora populations, participating in collaborative telemedicine and AI-driven initiatives tailored to ethnically diverse populations (9, 10).

We must also reframe how innovation is introduced in LMICs. Donations of diagnostic equipment without maintenance contracts, or algorithms trained exclusively on high-income population data, while worth evaluating as methods to increase the pace of diagnosis and care in underserved environments, could do more harm than good (11). Caribbean nations, like many LMICs, need investment in infrastructure, not just gadgets. Digital health strategies such as tele-epilepsy platforms, wearable seizure monitors or AI-powered triage systems, must be developed and tested with input from local clinicians, ensuring they reflect our unique epidemiology, cultural norms and even more mundane logistical issues such as broadband internet access (12, 13).

Equally essential is valuing the experience of senior neurologists. In both Canada and the Caribbean, late-career specialists represent a vital but underutilised resource. They are repositories of decades of clinical and institutional knowledge and are often eager to continue in part-time, teaching or advisory roles. Retaining this expertise through phased retirement schemes, academic partnerships and cross-regional mentorship programmes would yield immediate dividends (6).

The World Health Organization has taken a step forward with its Intersectoral Global Action Plan on Epilepsy and Other Neurological Disorders (14). However, without practical, funded implementation in LMICs and genuine representation of LMIC professionals in decision-making, these initiatives risk becoming more symbolic than transformational as envisaged.

A critical component of solving this crisis is aligning neurology policy with broader national development and financing priorities (15–17). Neurology must be positioned as central to ageing policy, chronic disease management and disability services. Integration into public health strategies, such as linking stroke prevention to cardiovascular programmes or embedding epilepsy services in maternal and child health, can improve both cost-efficiency and patient outcomes (18). Furthermore, ministries of finance and planning must be made to understand that underinvestment in neurological care leads to higher long-term costs due to lost productivity, increased dependency and repeated hospitalisations. Building this economic argument is essential for securing sustainable funding. In brief, brain health is population health. Canada's health-financing models offer instructive lessons, including bundled care, regional integration, and incentives for rural deployment (19).

Neurologists—indeed physicians in general—in the Caribbean and other LMICs must be seen not as passive recipients of aid or training but as co-creators of solutions. Only by engaging these professionals as equal partners through shared research networks, policy advisory roles, and regional leadership will we build sustainable neurological systems as is now so essential around the world (5, 20).

In summary, the shortage of neurologists is a global crisis with local consequences. It impairs not only the national capacity to manage acute neurological emergencies but also undermines long-term public health gains through inadequate stroke rehabilitation, epilepsy management, and dementia care. Multisectoral solutions combining education, infrastructure, digital innovation and sustained financing must be urgently pursued if we are to ensure equitable access to neurological care in the years to come. Its solution demands a new kind of partnership: one that bridges borders, centers equity, and values lived experience as much as technology. Especially in this time of changing opportunities and increasing isolationism, the Caribbean and Canada may be ideally positioned to lead such a model.

REFERENCES

1. Burton A. How do we fix the shortage of neurologists? Lancet Neurol 2018; **17(6)**: 502–3.
2. Abulaban AA, Obeid TH, Algahtani HA, Kojan SM, Al-Khathaami AM, Abulaban AA et al. Neurophobia among medical students. Neurosciences (Riyadh) 2015; **20**: 37–40.
3. Eberts M, Capurro D. Patient and physician perceptions of the impact of electronic health records on the patient–physician relationship. Appl Clin Inform 2019; **10**: 729–34.
4. Ali A. thebmjopinion [Internet]. BMJ2018. [cited 2024]. Available from: https://blogs.bmj.com/bmj/2018/07/27/amza-ali-overcoming-bias-against-research-from-low-income-countries/.
5. Ali A. Acknowledging and addressing bias towards research from lower and middle- income countries. West Indian Med J 2024; **71**: 1–2.
6. Busis NA, Shanafelt TD, Keran CM, Levin KH, Schwarz HB, Molano JR et al. Burnout, career satisfaction, and well-being among US neurologists in 2016. Neurology 2017; **88**: 797–808.
7. McGinley MP, Harvey T, Lopez R, Ontaneda D, Buchalter RB. Geographic disparities in access to neurologists and multiple sclerosis care in the United States. Neurology 2024; **102**: e207916.
8. Ahmed SN, Wiebe S, Mann C, Ohinmaa A. Telemedicine and epilepsy care—a Canada wide survey. Can J Neurol Sci 2010; **37**: 814–8.
9. Mandl KD. How AI could reshape health care-rise in direct-to-consumer models. JAMA 2025; **333**: 1667–69.
10. Harding L, McFarlane J, Honey CR, McDonald PJ, Illes J. Mapping the landscape of equitable access to advanced neurotechnologies in Canada. Can J Neurol Sci 2023; **50**: s17–25.

11. Beniczky S, Wiebe S, Jeppesen J, Tatum WO, Brazdil M, Wang Y et al. Automated seizure detection using wearable devices: a clinical practice guideline of the International League against epilepsy and the International Federation of Clinical Neurophysiology. Clin Neurophysiol 2021; **132**: 1173–84.
12. Brinkmann BH, Karoly PJ, Nurse ES, Dumanis SB, Nasseri M, Viana PF et al. Seizure diaries and forecasting with wearables: epilepsy monitoring outside the clinic. Front Neurol 2021; **12**: 690404.
13. Khadilkar SV. Neurology in India. Ann Indian Acad Neurol 2013; **16**: 465–6.
14. Grisold W, Freedman M, Gouider R, Guekht A, Lewis S, Medina M et al. The Intersectoral Global Action Plan (IGAP): a unique opportunity for neurology across the globe. J Neurol Sci 2023; **449**: 120645.
15. Ellis RP. Financing Health Care: New Ideas for a Changing Society 2008.
16. Ellis RP. Risk adjustment in health care markets: concepts and applications. Financing Health Care 2008: 177–222.
17. Mateen FJ. Progress towards the 2030 sustainable development goals: direct and indirect impacts on neurological disorders. J Neurol 2022; **269**: 4623–34.
18. Pauranik A. Advocacy in neurology. Ann Indian Acad Neurol 2008; 11: 60–5.
19. Kirby S, Weston LE, Barton JJ, Buske L, Chauhan TS. Report of the Canadian Neurological Society Manpower Survey 2012. Can J Neurol Sci 2016; **43**: 227–37.
20. Janca A, Aarli JA, Prilipko L, Dua T, Saxena S, Saraceno B. WHO/WFN survey of neurological services: a worldwide perspective. J Neurol Sci 2006; **247**: 29–34.

ORIGINAL ARTICLE

Determining the Sensitivity and Specificity of Electroneuromyographic Tests in Patients with the Prediagnosis of Ulnar Neuropathy at the Elbow

PD Analan, B Leblebici

ABSTRACT

Objective: *To assess the sensitivity and specificity of nerve conduction studies (NCS) in patients pre-diagnosed with ulnar neuropathy at the elbow (UNE).*

Method: *This study assessed 166 extremities of 124 patients (68 females and 56 males) using electroneuromyographic (ENMG) testing. Nerve conduction velocity (NCV), sensory nerve action potential (SNAP) amplitudes, and compound motor action potential (CMAP) amplitudes were obtained by stimulating the abductor digiti minimi (ADM) and first dorsal interosseous (FDI) muscles. Patients were divided into two groups based on the diagnosis made after ENMG testing; the UNE group (11 patients) and the non-UNE group (114 extremities of 84 patients). The remaining 41 extremities (15 females and 14 males) with non-UNE ENMG pathologies were excluded from the dataset. Sensitivity and specificity values of the motor nerve conduction studies at the ADM and FDI muscles were determined. Nerve conduction velocity of the forearm segment of the ulnar nerve was subtracted mathematically from the NCV of the elbow segment to determine the effectiveness of the forearm–elbow inter-segmental velocity difference (FIVD) for the diagnosis of UNE.*

Result: *Agreement between the pre-diagnosis and the electrophysiological diagnosis of UNE was 6.7%. Ulnar CMAP amplitudes of the ADM at the elbow segment and SNAP amplitudes at the elbow segment were similar (*$p > 0.05$*). Reduced CMAP amplitude had 100% sensitivity and specificity. Sensitivity and specificity rates for NCV from the FDI were 99.1 and 81.8%, respectively, and 98.2 and 90.9%, respectively, from the ADM. Sensorial, ADM and FDI FIVD sensitivity rates were 81.8%, 81.8% and 72.7%, respectively, and specificity rates were 86.8%, 78.1% and 90.2%, respectively.*

Conclusion: *Agreement between clinical pre-diagnosis and ENMG diagnosis of UNE was very low. However, the determination of reduced amplitude and NCV in the elbow segment of the ulnar nerve using ENMG had high sensitivity and specificity. In such patients, FIVD may be useful in diagnosing UNE.*

Keywords: Electroneuromyography, sensitivity and specificity, ulnar neuropathy at the elbow.

INTRODUCTION

The ulnar nerve enters the ulnar groove formed between the medial epicondyle and the olecranon process at the elbow. The ulnar nerve travels slightly distal to the groove in the proximal forearm under the tendinous arch of the two heads of the flexor carpi ulnaris (FCU) muscle, known as the cubital tunnel. Entrapment of the ulnar nerve in this region is known as ulnar neuropathy at the elbow (UNE) (1).

In patients with UNE, the clinical picture ranges from intermittent paresthesias in the fourth and fifth digits to complete sensory loss in the territory of the ulnar nerve with atrophy and weakness of the ulnar muscles. As the majority of the intrinsic hand muscles are

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ulnar innervated, weakness of these muscles leads to loss of dexterity and decreased grip and pinch strength (1, 2). Ulnar neuropathy at the elbow is the second most common entrapment neuropathy after carpal tunnel syndrome (CTS).

Electroneuromyography (ENMG) tests are commonly used for the diagnosis of UNE. In addition, ENMG can be used to determine nerve dysfunction and its severity (1). In 1999, the American Association of Electrodiagnostic Medicine (AAEM) published ENMG application protocols for UNE (3, 4). These ENMG protocols include sensory and motor nerve conduction studies (NCS) and needle electromyography (EMG) applications. Motor NCS can be made by recording from the abductor digiti minimi (ADM) and/or first dorsal interosseous (FDI) muscles with ulnar nerve innervations (1). No international agreement has been made concerning the sensitivity and specificity of electrodiagnostic studies for the diagnosis of UNE. Sensitivity rates have been reported between 37% and 86% and specificity starting at 95% (2). However, motor NCS tests have shown different sensitivities and specificities in the literature (2, 4, 5, 9, 12–14). Some studies have reported that ENMG of the FDI is more sensitive, others have suggested that ADM recordings alone are adequate and yet others advocate taking both recordings. Despite these various application possibilities, diagnosis may not be verified in some patients. The results of one electrodiagnostic study showed that 16% of patients with electrophysiological UNE were not diagnosed in evaluations using only two stimulation sites below and above the ulnar groove (5).

The present study aims to assess the sensitivity and specificity of ENMG studies in patients pre-diagnosed with UNE.

SUBJECTS AND METHOD

This study was approved by the local ethics committee and written informed consent was obtained from all participants. Power analysis during the bio-statistical preliminary assessment indicated a study population of 137 patient extremities. Consequently, this prospective study included 166 upper extremities of 124 sequential patients (68 females and 56 males) referred to our ENMG Clinic.

Inclusion criteria were clinical pre-diagnosis of UNE in addition to the presence of paresthesia, weakness or pain in the ulnar nerve territory which worsened or became more prominent at elbow flexion, and positive Tinel's sign at the ulnar groove.

Exclusion criteria were neurological conditions such as stroke, focal or generalised neuropathies, brachial plexus lesions, lesions of the ulnar nerve proximal or distal to the elbow, a history of upper extremity surgery, trauma or corticosteroid injections for treatment of these symptoms, and inflammatory or rheumatologic disorders that potentially affected nerve conduction such as rheumatoid arthritis or gout. Pregnancy, systemic disorders affecting peripheral nerves such as diabetes mellitus or chronic renal failure, and any abnormalities found via NCS were also reasons for exclusion.

Patients were divided into two groups based on diagnosis made after ENMG testing. The UNE group included 11 patients (six females and five males) and the non-UNE group consisted of 114 extremities of 84 patients (47 females and 37 males). The remaining 41 extremities of 29 patients (15 females and 14 males) who had non-UNE ENMG pathologies were excluded from the dataset and statistical evaluation was performed based on the two groups.

Nerve Conduction Studies

All tests were performed using a four-channel Medelec Synergy ENMG system (Oxford Instruments Medical, Surrey, England).

If the hands of the patients were cool they were warmed by a hot pack before testing. Patients were seated on an exam chair and a pillow placed on the lap to relax the hands. Motor NCS was performed using superficial disc electrodes (Ag/AgCl) of 10 mm diameter and sensory NCS using ring-shaped recording electrodes. Stimuli were provided by bipolar electrodes with a distance of 25 mm between the ends and 6 mm diameter. The distance between active and reference electrodes in all ENMG evaluations was 4 cm. A metal ground electrode was placed between the active and stimulating electrodes.

Motor orthodromic and sensory antidromic studies of the ulnar and median nerves were performed on the extremities of complaint of all patients included in the study. In addition, median nerve palm–wrist segment NCS were performed in order to assess isolated UNE. In patients with non-UNE ENMG pathologies, further ENMG testing was scheduled for diagnosis. Ulnar NCS was performed with the elbow at 70–90° flexion in order to prevent subluxation of the nerve in the ulnar sulcus. For motor nerve conduction studies, the recording electrode was placed at the belly points of the ADM and FDI muscles. The reference electrode for the ADM stimulus was placed immediately distal to the

5th metacarpophalangeal (MCP) joint and at the middle of the thumb for the FDI. Stimuli were given from the wrist, 4–6 cm below and 6–8 cm above the midpoint of the ulnar sulcus at the elbow, the axilla and at the Erb's point. When giving stimulus from the wrist to the ADM, the distance between the recording electrodes was adjusted to 8 cm. The stimulation of the FDI from the wrist was performed over the FCU tendon at the proximal wrist line level.

The ulnar sensory NCS, the cathode ring electrode was placed at the 5th MCP joint and the anode electrode at the 5th distal interphalangeal joint. The distance between the two points was arranged to give stimuli from 12 cm proximal to the cathode ring electrode at the proximal. The other stimulation points were located 4–6 cm below and 6–8 cm above the midpoint of the ulnar sulcus at the elbow.

In the median NCS, the sensory latency was recorded from the third MCP joint stimulated at a point 12 cm proximal to the recording electrode. Motor NCS was measured from the mid portion of the abductor pollicis brevis (APB) muscle and by the stimulation of the median nerve 8 cm proximal to the recording electrode. Proximal stimulations were performed in the medial epicondylar area.

For the median palm–wrist segment NCS, the active superficial disc recording electrode was placed at the proximal palm–wrist segment line and the reference electrode was placed at 4 cm proximal to the active electrode. The anode part of the stimulator was placed on the 2nd MCP joint and the cathode was placed in the palm to face the wrist.

Mixed, motor and sensory nerve conduction velocities (NCV), motor distal latencies (MDL), sensory distal latencies, compound muscle action potential (CMAP) amplitudes, sensory nerve action potential (SNAP) amplitudes, and F-wave latencies were recorded.

For sensory distal latencies, the point at which the SNAP amplitude peaked was taken as the reference and the first separation point from the isoelectric line at the negative direction for the MDL. For the CMAP and SNAP amplitudes, the measurement between the first negative peak point of the action potential and the isoelectric line was recorded.

The same type and size of electrodes were used for all patients. Filter settings were from 3 Hz to 10 kHz for motor NCS and from 20 Hz to 2 kHz for sensory NCS. In order to obtain the most optimal result in the NCS, all stimuli were given supramaximally. To minimise the technical factors and optimise the results, a minimum of eight recordings were obtained for the sensorial NCS. The average of these results was accepted as the mean result. For motor NCS, at least three recordings were obtained and the best result was recorded.

Electrodiagnostic criteria

Diagnoses were made using AAEM recommendations (3) and parameters developed in the local ENMG laboratory.

Accordingly, for stimulus from the ADM muscle, the following values were considered normal: ulnar nerve MDL < 3.45 ms, CMAP amplitude > 4.32 mV, NCV > 53.6 m/s, F-wave latency < 28.41 ms, ulnar nerve sensory distal latency < 3.41 ms, SNAP amplitude > 11 mV, and NCV > 54.2 m/sn. With stimulus from the FDI muscle, stimuli limits were determined as follows: MDL < 3.40 ms, CMAP amplitude > 4.30 mV and NCV > 54 m/s.

For the median nerve, the following results were assumed as normal with stimulus from the APB muscle: MDL < 4.00 ms, CMAP amplitude > 4.25 mV, NCV > 50.3 m/s, sensory distal latency < 3.41 ms, SNAP amplitude > 10 mV, NCV > 50.6 m/s, mean F-wave latency < 27.87 ms. In the median nerve mixed NCS, NCV at the palm–wrist segment of > 35.9 m/s and distal amplitude > 32.4 mV were considered normal.

Electrodiagnostic criteria for the UNE as measured by the motor NCS were as follows: NCV at the elbow segment of the ulnar nerve < 50 m/s, amplitude < 20% that of the amplitude obtained with stimulus from the wrist, mean F-wave latency > 28.41 ms, sensory NCV < 48 m/s, and SNAP amplitude < 2 mV. Amplitude of the elbow segment 20% lower than the forearm segment was considered conduction block and reduced NCV of the same segment focal demyelination.

Differences between the NCV of the forearm and elbow segments were evaluated as reported in Shakir *et al*. (6). The forearm–elbow inter-segmentary velocity difference (FIVD) was calculated for both sensory and motor NCS as the NCV of the forearm segment was mathematically subtracted from the NCV of the elbow segment. Needle EMG was applied to the FCU, ADM and FDI muscles of patients diagnosed with UNE according to the criteria given above. In the presence of denervation potentials, motor unit action potentials and recruitment pathologies were recorded. Abnormal needle EMG findings were considered axonal damage. Needle EMG was conducted in muscles other than the ADM, FDI and FCU for the differential diagnosis when necessary.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS software (Version 17.0, SPSS Inc., Chicago, IL, USA). The Kolmogorov Smirnov and Shapiro–Wilk tests and histograms were used to confirm the normality of each continuous variable. Categorical variables between groups were analysed using the Chi-square test or Fisher's exact test.

The Student's *t*-test for the normally distributed data was used for the comparisons between groups. SPSS statistical software17.0 (SPSS Inc.) was used to construct the receiver operating characteristic curves and calculate the areas under curve (AUC), sensitivity and specificity. *p* values of less than 0.05 were considered statistically significant.

RESULTS

Table 1 summarises diagnoses resulting from ENMG findings.

Table 1: Electoneuromyographic findings

Electoneuromyographic impressions	N	%
Normal	114	69.09
UNE	11	6.67
Non-localizable ulnar neuropathy	13	7.8
CTS + UNE	8	4.8
CTS	6	3.6
UNE + CTS + PNP	4	2.4
CTS + PNP	3	1.8
Non-localizable ulnar neuropathy + CTS	2	1.2
CTS + UNW	2	1.2
Mononeuritis multiplex	1	0.6
UNW	1	0.6
Motor neuron disease	1	0.6
Total		**166**

UNE = ulnar neuropathy at the elbow; UNW = ulnar neuropathy at the wrist; CTS = carpal tunnel syndrome; PNP = polyneuropathy.

Ulnar neuropathy at the elbow was identified in only 11 of the 166 extremities. Electroneuromyographic data of 114 patient extremities were completely normal. The remaining 41 extremities, which had results consistent with non-UNE ENMG pathologies, were not included in the results. Agreement between the pre-diagnosis and the electrophysiological diagnosis was 6.7% and the kappa coefficient was 0%.

Gender, age, height, weight, and body mass index of the two groups were similar ($p > 0.05$).

F-wave latencies, sensory and motor NCV, all FIVD measurements, and CMAP amplitudes from the FDI muscle at the elbow segment were statistically different between the two groups ($p < 0.05$). Distal latencies, CMAP amplitudes from the ADM muscle and SNAP amplitudes from the elbow segments were similar ($p > 0.05$). Data of the two groups are Summarised in Table 2.

In the UNE group, reduction of amplitude lower than 20% at the elbow segment from the stimulus at the ADM, and FDI was found to be in 100% kappa agreement. For this evaluation, the sensitivity and specificity were 100%.

The UNE probability was determined to be 90.6% when the NCV value from FDI muscle was lower than 54.9 m/s with a sensitivity of 99.1% and specificity of 81.8%. For the ADM, the rate of accuracy was 93.8% with a cut-off value of 53.65 m/s. Sensitivity and specificity were 98.2 and 90.9%, respectively.

When sensorial FIVD was greater than 10.9 m/s, the probability of UNE was 88.9% at a sensitivity of 81.8% and specificity of 86.8%. When the FIVD from stimulus at the ADM muscle was greater than 6.8 m/s, the probability of UNE was 80.1% with a sensitivity of 81.8% and specificity of 78.1%. The cut-off value of FIVD for the FDI muscle was 8.4 m/s with a UNE probability of 86.9%. The sensitivity and specificity for this evaluation were 72.7 and 90.2%, respectively.

The other ENMG parameters sensitivity and specificity could not be calculated due to the lack of kappa agreement.

Data of patients diagnosed with isolated UNE were statistically evaluated. Pathological ENMG findings of the UNE group can be found in Table 3.

DISCUSSION

Our ENMG results were within normal limits and the agreement between clinical pre-diagnosis and ENMG results was 6.7%. Studies conducted in Turkey have reported a clinical pre-diagnosis-ENMG impressions agreement rate between 42.3 and 55% (7).

The extremely low agreement between clinical pre-diagnosis and ENMG results may suggest that inadequate clinical pre-diagnosis routinely leads to unnecessary ENMG requests. However, such a generalisation may not be correct. A study evaluating 130 cadavers reported that anatomical variations may create difficulty in determining the causes of UNE (8). Within a peripheral nerve, nerve fibres innerving certain muscles are separated into different fascicles by the connective tissue. In the ulnar nerve, the nerve fibre bundle innerving the ADM muscle may be different than that innerving the FDI muscle and these nerve fibre bundles may be localised at different

Table 2: Clinical and electroneuromyographic characteristics of groups (mean ± standard deviation)

	Group 1 (UNE)	Group 2 (non-UNE)	*p*
Clinical characteristics			
Age (years)	41.2 ± 13.78	42.52 ± 12.41	0.753
Body length (cm)	168.4 ± 7.56	162.32 ± 27.67	0.493
Weight (kg)	73.1 ± 18.14	74.15 ± 13.12	0.82
Body mass index (kg/m^2)	25.54 ± 5.04	26.74 ± 4.31	0.42
	n (11)		*%*
Electroneuromyographic results			
Minimum F wave latency (ms)	26.31 ± 2.29	24.89 ± 1.62	**0.009**
Mean F wave latency (ms)	27.54 ± 2.16	25.95 ± 1.69	**0.005**
Ulnar nerve sensory measurements			
Distal latency (ms)	2.85 ± 2.22	2.69 ± 0.31	0.107
SNAP Amplitude at the elbow (mV)	11.91 ± 8.25	16.36 ± 8.71	0.107
NCV at the elbow segment (m/s)	46.54 ± 9.89	70.06 ± 10.84	**0.0**
NCV at the forearm segment (m/s)	64.81 ± 6.4	65.7 ± 6.42	0.661
FIVD	18.27 ± 12.35	−4.36 ± 14.57	**0.0**
Ulnar nerve motor measurements of abductor digit minimi muscle			
Distal latency (ms)	2.61 ± 0.34	2.58 ± 0.24	0.752
CMAP Amplitude at the elbow (mV)	7.8 ± 2.17	8.96 ± 2.42	0.131
NCV at the elbow segment (m/s)	49.6 ± 10.07	67.58 ± 9.21	**0.0**
NCV at the forearm segment (m/s)	63.31 ± 7.62	64.96 ± 5.62	0.371
FIVD	13.7 ± 15.26	−2.61 ± 11.26	**0.0**
Ulnar nerve motor measurements of first dorsal interosseous muscle			
Distal latency (ms)	3.0 ± 0.32	3.04 ± 0.27	0.598
CMAP Amplitude at the elbow (mV)	6.02 ± 2.45	7.73 ± 2.63	**0.042**
NCV at the elbow segment (m/s)	47.989.44	66.39 ± 9.05	**0.0**
NCV at the forearm segment (m/s)	59.9 ± 5.13	61.52 ± 5.77	0.370
FIVD	11.92 ± 11.91	−4.86 ± 10.94	**0.0**

NCV = nerve conduction velocity; CMAP = compound muscle action potential; SNAP = sensory nerve action potential; FIVD = forearm-elbow inter-segmental velocity difference.

Table 3: Classification of pathological electroneuromyographic findings in the ulnar neuropathy at the elbow group

Axonal damage based on needle EMG		
FDI	3	27.27
ADM	3	27.27
FCU	3	27.27
Conduction block		
Ulnar nerve/sensory	4	36.36
Ulnar nerve/FDI motor	1	9.09
Ulnar nerve/ADM motor	1	9.09
Focal demyelination		
Ulnar nerve/sensory	8	72.72
Ulnar nerve/FDI motor	9	81.81
Ulnar nerve/ADM motor	7	62.63

ENMG = electroneuromyography; UNE = ulnar neuropathy at the elbow; ADM = muscle of abductor digiti minimi; FDI = muscle of first dorsal interosseous; FCU = muscle of flexor carpi ulnaris.

regions of the ulnar nerve. Thus, external pressure on the peripheral nerve may result in clinical and electrophysiological heterogeneity depending on the anatomical localisation at the site of pressure and the damage of the fascicles of the nerve (1, 2, 9).

Stewart *et al*. (9) demonstrated that ulnar NCV in the same individual can vary by as much as 40 m/s. Such inaccuracies are likely the result of several factors: (a) depolarisation of the nerve some variable distance from the electrode, (b) shifts in electrode position due to skin laxity, and (c) the disproportionate effect of small errors in measurement due to the short distance between the proximal and distal stimulation sites. Our results may have been affected by one or more of these factors.

Azma *et al*. (10) compared the normal ulnar motor NCV values using the FDI and ADM on 100 nerves of healthy volunteers. Mean NCVs at the elbow were 62.65 ± 7.62 m/s and 60.49 ± 7.42 m/s for the ADM and FDI, respectively. Similarly, in the current study, NCV was 67.58 ± 9.21 m/s for the ADM and 66.39 ± 9.05 m/s for the FDI in the non-UNE group.

Padua *et al*. reported that false negatives were common with ENMG in cases of mild UNE (11). In

the present study, CMAP amplitudes of the ADM and SNAP amplitude were similar between the two groups. Accordingly, false negatives may have occurred with mild UNE present in patients in the non-UNE group with reduced amplitude and without an obvious conduction block which could not be identified.

While the pathophysiology of many cases of UNE is of axonal loss, NCV demonstrates only a non-localisable ulnar neuropathy (1). In the present study, non-localisable ulnar neuropathy was detected in 13 cases. However, ENMG assessments of these 13 patients were not sufficient to verify the UNE co-existing with axonal loss.

Studies in the literature have compared ENMG results from the ADM and FDI muscles of UNE patients. Kothari *et al*. (12) found that motor NCS of the FDI and ADM was abnormal in 81 and 71% of patients, respectively. Caliandro *et al*. (2) showed that FDI-innervated ulnar fibres have a higher susceptibility to damage than those of the ADM and reported sensitivity and specificity rates of 58 and 100%, respectively, between the NCVs of both the FDI and ADM. Other studies have made similar findings (9, 13). Another study has suggested that recording from the FDI was not routinely indicated and that ADM recordings were sufficient for the diagnosis of UNE (5). Conversely, other studies argued that the ADM and FDI muscles were equally sensitive (4, 14). In the present study, a high rate of sensitivity and specificity was found in the FIDV, CMAP amplitude and motor NCV at the elbow segment. These sensitivity and specificity values were close for both muscles.

Kothari *et al*. (12) observed that the most common localizing finding of ENMG was conduction velocity slowing at the elbow segment. Findings in the current study (rate of slowing of NCV: 81.8%) most resemble those of Kothari *et al*.

The FCU muscle may also be affected in UNE in agreement with its clinical and electrophysiological severity (1). In the present study, the determination rate of FCU impact by needle EMG was 27.27%.

Forearm-elbow inter-segmental velocity difference may be used in the diagnosis of UNE, with differences greater than 10 m/s signalling UNE (3). Shakir *et al*. (6) compared motor NCV and FIVD of the ADM and FDI muscles of 85 subjects with UNE and 77 subjects with CTS without clinical evidence of UNE. At 95% specificity, motor NCV sensitivities were 80% at the ADM and 77% at the FDI, while FIVD sensitivities were 51% and 38%, respectively. In the current study, FIVD was evaluated in both sensorial and motor measurements and sensorial and motor FIVDs had high sensitivity and specificity rates. However, while Shakir *et al*. divided the UNE group into three subgroups based on the CMAP amplitude, we were unable to divide our groups based on CMAP amplitude and this difference in methodology should be considered when comparing these two studies.

The low number of UNE patients in accordance with the ENMG criteria can be considered a limitation of the present study and may affect the sensitivity and specificity reliability. In addition, the high number of patients with normal ENMG findings and the comparison between these two groups may have affected the present results. Studies with a higher number of patients with UNE diagnosis may contribute further to the literature.

In conclusion, the proportion of patients diagnosed with UNE that are in accordance with the existing electroneuromyographic criteria appears to be very low. According to our data, reduced CMAP amplitude has 100% sensitivity and specificity. In addition, elbow segment NCV from the ADM and FDI muscles have high sensitivity and specificity. However, sensitivity and specificity values were similar for the ADM and FDI muscles. Sensory and ADM/FDI FIVD may be useful for the diagnosis of UNE.

REFERENCES

1. Preston DC, Shapiro B. Electromyography and neuromuscular disorders, clinical-electrophysiologic correlations. Philadelphia: Elsevier; **2005:** 291–312.
2. Caliandro P, Foschini M, Pazzaglia C, La Torre G, Aprile I, Granata G et al. IN-RATIO: a new test to increase diagnostic sensitivity in ulnar nerve entrapment at elbow. Clin Neurophysiol 2008; **119:** 1600–6.
3. American Association of Electrodiagnostic Medicine. Practice parameter for electrodiagnostic studies in ulnar neuropathy at the elbow: summary statement. American Association of Electrodiagnostic Medicine, American Academy of Neurology, American Academy of Physical Medicine and Rehabilitation. Muscle Nerve 1999; **22:** 408–11.
4. Beekman R, Van Der Plas JP, Uitdehaag BM, Schellens RL, Visser LH. Clinical, electrodiagnostic, and sonographic studies in ulnar neuropathy at the elbow. Muscle Nerve 2004a; **30:** 202–8.
5. Todnem K, Michler RP, Wader TE, Engstrøm M, Sand T. The impact of extended electrodiagnostic studies in ulnar neuropathy at the elbow. BMC Neurol 2009; **9:** 52.
6. Shakir A, Micklesen PJ, Robinson LR. Which motor nerve conduction study is best in ulnar neuropathy at the elbow? Muscle Nerve 2004; **29:** 585–90.
7. Yağcı I, Ofluoglu D, Gündüz H, KaradağSaygı E, Güven Z, Berker N et al. The consistency of clinical referral diagnosis and electrophysiological diagnosis in pediatric cases. Turk J Phys Med Rehab 2008; **54:** 92–5.
8. Campbell WW, Pridgeon RM, Riaz G, Astruc J, Sahni KS. Variations in anatomy of the ulnar nerve at the cubital tunnel: pitfalls in the diagnosis of ulnar neuropathy at the elbow. Muscle Nerve 1991; **14:** 733–8.
9. Stewart JD. The variable clinical manifestations of ulnar neuropathies at the elbow. J Neurol Neurosurg Psychiatry 1987; **50:** 252–8.
10. Azma K, Bahmanteimoury K, Tavana B, Moghaddam FR, Moghaddam NM, Mahmoudi H. Two measurement methods of motor ulnar nerve conduction velocity at the elbow: a comparative study. Neurol India 2007; **55:** 145–7.

11. Padua L, Aprile I, Mazza O, Padua R, Pietracci E, Caliandro P et al. Neurophysiological classification of ulnar entrapment across the elbow. Neurol Sci 2001; **22:** 11–6.
12. Kothari MJ, Heistand M, Rutkove SB. Three ulnar nerve conduction studies in patients with ulnar neuropathy at the elbow. Arch Phys Med Rehabil 1998; **79:** 87–9.
13. Dumitru D, Zwarts MJ. Focal Peripheral Neuropathies. In: Dumitru D, Amato AA, Zwarts MJ, eds. Electrodiagnostic medicine. Hanley and Belfus Inc: Philadelphia; **2002:** 1076–81.
14. Tackmann W, Vogel P, Kaeser HE, Ettlin T. Sensitivity and localizing significance of motor and sensory electroneurographic parameters in the diagnosis of ulnar nerve lesions at the elbow. J Neurol 1984; **231:** 204–11.

ORIGINAL ARTICLE

No Role of rs1137101 Variant of the Leptin Receptor Gene in Manifestation of Obesity

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ABSTRACT

Objective: *Obese phenotype can be a consequence of disruption in the physiological integrity of the leptin axis. Leptin acts as a satiety signal and exerts its physiological effects by binding to the leptin receptor; consequently, genetic variants of the leptin receptor may be important in the pathophysiology of human obesity. The current study was therefore carried out to find the association of the leptin receptor rs1137101 variant with obesity and associated anthropometric and metabolic parameters in a sample of the Pakistani population.*

Methods: *DNA samples from a total of 239 obese and 155 non-obese human subjects with age range of 5–45 years were genotyped for the rs1137101 variant of the leptin receptor gene. Anthropometric measurements of the subjects were taken, and biochemical parameters from the corresponding serum samples were determined. Blood pressure was monitored, and measurements of body weight, height, waist and hip circumference were recorded. Body mass index and waist-to-hip ratio were calculated. Levels of fasting blood glucose, insulin, leptin and leptin receptor were determined, and insulin resistance was calculated.*

Results: *The statistical analysis of the data showed no significant difference in genotype and allele frequencies of rs1137101 variant between obese and non-obese subjects (*$p > 0.05$*). Moreover, no significant association of the variant was observed with any of the obesity-related anthropometric and metabolic traits (*$p > 0.05$*).*

Conclusion: *The study suggests that rs1137101 variant of the leptin receptor gene may not be involved in conferring obese phenotype in the Pakistani population.*

Keywords: Leptin receptor, obesity, Pakistan, rs1137101 variant.

INTRODUCTION

Leptin receptor (LEPR) is a single transmembrane protein that belongs to the class I cytokine receptor family (1). Leptin, an adipokine and central regulator for adiposity-sensing pathways, exerts its physiological effects by binding to LEPR in the hypothalamus. It functions as a satiety signal by downregulating orexigenic (appetite-stimulating) peptides and upregulating anorexigenic (appetite-decreasing) peptides (2). Leptin receptor is a product of the LEPR gene that is located on human chromosome 1p31 and consists of 20 exons (3). The expression of the LEPR gene results in at least six isoforms of leptin receptors (LEPRa-f) attributable to alternative mRNA splicing. The isoforms (Ra, Rb, Rc, Rd, and Rf) share the same extracellular and transmembrane domains but differ in the length of the intracellular domain (4).

The secreted isoform Re lacking both transmembrane and intracellular domains can be generated either by alternative splicing (Ob-Re) or by ectodomain shedding. It circulates as a soluble receptor, and may be involved in modulating leptin activity (5, 6). Leptin

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receptors form homodimers, and binding of leptin to the receptors activates Janus-activated kinases (JAK), which in turn activate signal transducers and activators of transcription (STAT). Leptin signalling through the JAKs and activation of transcription system for regulating body fat mass is mainly associated with the longest form (LEPRb) of LEPR that contains both conserved sequence motifs necessary for the binding of the JAKs and STAT family of signal transducing factors required for full leptin signalling function (7). The shorter isoforms lack STAT binding elements, thus having reduced signal transduction capabilities (8). The longest isoform appears to be the most abundant in the hypothalamus, while the shorter isoforms seem to predominate in peripheral tissues (9).

The LEPR gene has been extensively studied in search for several common variants that may be important in the pathophysiology of human obesity (10). One of these variants is non-synonymous Q223R (rs1137101) in exon 6 of the human LEPR gene at codon 223 (11). The LEPR contains two extracellular cytokine receptor homology domains (CRH1 and CRH2). The CRH2 domain is necessary and sufficient for leptin binding, whereas CRH1 is not essential for a high affinity interaction (12). The Q223R variant results from an A to G transversion at nucleotide 668 from the start codon encoding a glutamine (Q) to arginine (R) substitution in the N-terminal CRH1 domain of LEPR (13). Genetic variants, which serve as causal factors for a particular population, may or may not affect another population. In particular, the data on the status of this variant in relation to obesity in the Pakistani population has remained extremely scarce. The current study was therefore undertaken to find the association of Q223R variant of the LEPR gene with obesity and obesity-related anthropometric and metabolic traits in a sample of the Pakistani population.

SUBJECTS AND METHODS

The study was performed at the University of Health Sciences Lahore, Pakistan, after the approval from the Institutional Review Board of the University. All procedures involving collection of blood samples and anthropometric measurements were carried out with the adequate understanding and written informed consent of the subjects.

Study population

The participants of the study involved 239 obese individuals with body mass index (BMI) ≥ 30 kg/m^2 or $> 95^{th}$ percentile and 155 control subjects with BMI < 25 kg/m^2 or 5^{th}–85^{th} percentile. The age range of the participants was between 5 and 45 years. Simple random sampling was applied for the collection of blood specimens. Subjects with a history of any endocrine disorder were not included in the study. Similarly, subjects who were taking antidepressants, anticonvulsants and steroids were not included in the study.

Categorisation of study population

Subjects were further classified into two categories: category 1 involving individuals ≤ 18 years of age and category 2 involving individuals > 18 years of age. The subjects > 18 years of age were considered obese with a BMI ≥ 30 and normal weight with a BMI < 25 (14). The subjects ≤ 18 years of age were considered obese if their BMI was $> 95^{th}$ percentile and normal weight if their BMI fell within the 5^{th}–85^{th} percentile by following BMI for age growth charts recommended by the Centers for Disease Control and Prevention.

Demographic information and physical examination

Full demographic information including name, age, sex, address, education and socio-economic status was documented on a questionnaire. A complete general physical examination was carried out. A blood volume of 5 ml was collected from each subject aseptically.

Anthropometric and blood pressure measurements

Body weight (BW), height, waist circumference (WC) and hip circumference (HC), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by standard procedures (14). Body height was measured using a stadiometer and BW was measured on digital weighing scale. The BMI of each subject was calculated as weight in kilograms divided by height in metres squared. Waist circumference was taken just above the belly button in the middle of the lower border of the costal margin and uppermost border of the iliac crest to the nearest 0.1 cm. Hip circumference was measured as the maximal circumference over the buttocks. Waist-to-hip ratio was calculated from the values of waist and hip circumference. Blood pressure was taken two times in a sitting position from the right arm of the subject by a standard mercury sphygmomanometer.

Biochemical measurements

Blood samples were collected after an overnight fast of 8–12 hours. Fasting blood glucose (FBG) levels were determined by the glucose oxidase method on Huma Star 180 chemistry analyser (Human, Wiesbaden, Germany).

Blood insulin, leptin and LEPR levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits on an automated EIA analyser (Bio-Rad Laboratories, Hercules, CA, USA). Fasting blood glucose and fasting insulin concentrations were taken to measure homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR = Fasting insulin (μ IU/ml) × Fasting glucose (mmol/L)⁄22.5 (15).

DNA extraction and genotyping of rs1137101 variant

DNA was extracted from whole blood using a genomic DNA purification kit (Fermentas, Waltham, MA, USA). Genotyping of rs1137101 variant was carried out by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism assay). A DNA fragment containing rs1137101 variant site was amplified using a specific forward (5′TAAGCTGGGTGTCCCAAATA3′) and reverse (5′AGCAAAGTGAGATAAGCT3′) primer-pair. The PCR was done on Icycler 5 (Bio-Rad). The PCR reaction mix was composed of 100 μg DNA, 1X Taq buffer, 2 mM MgCl2, 0.2 mM of each dNTP, 10 μmol of each primer and 0.5 U Taq DNA polymerase in a final volume of 25 μl. The PCR conditions involve initial denaturation at 95°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute, and then a final extension step at 72°C for 10 minutes. MspI restriction enzyme (Favorgen, Taiwan) was used to digest amplified products of 424 bp in order to examine rs1137101 variant by RFLP assay. Presence of the A allele generated a single fragment of 424 bp, whereas presence of the G allele revealed two fragments of 285 and 139 bp (Figure)

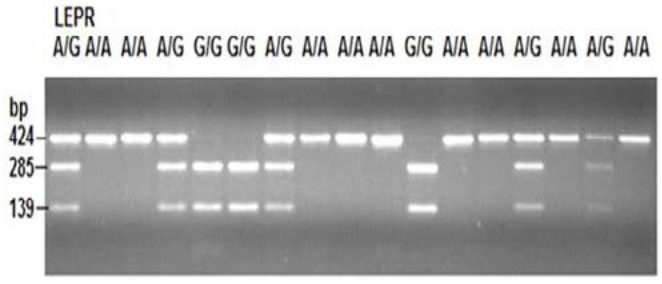

Figure: Restriction fragment length polymorphism (RFLP) analysis of rs1137101 variant in the LEPR gene.

Statistical analysis

The Statistical Package for the Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA) software was used for analysing the data. Quantitative variables were expressed as mean ± standard error of mean (SEM). Student's *t*-test was applied to observe the differences between cases and controls. The whole data were stratified according to age and sex into subcategories.

Hardy–Weinberg equilibrium test was applied to determine the variation in distribution of alleles and genotypes. Allelic frequencies were calculated by gene counting. Chi-square test was used to determine the significant differences in genotype and allelic frequencies between study and control groups. Association of rs1137101 variant (A>G transition) with obesity was determined by Pearson Chi-square using co-dominant, dominant and recessive models. Odds ratio (OR) and 95% confidence intervals (CI) were calculated to determine the risk of obesity associated with the risk allele. The association of rs1137101 variant with anthropometric and metabolic traits was determined using a general linear model assuming co-dominant, dominant and recessive genetic models. The genotypes were coded as (0, 1 and 2) in co-dominant model, (0 and 1) in dominant model and (1, 0) in recessive model corresponding to the number of copies of risk allele. Analysis of variance, Tukey post hoc analysis and *t*-test were applied to test the differences of obesity-related anthropometric and metabolic traits across genotypes of rs1137101 variant adjusted for age and sex. Bonferroni adjustment was carried out for multiple comparisons. A p-value of < 0.05 was considered statistically significant.

RESULTS

Obese subjects and high-risk factors for cardiometabolic disorders

In our study, family history of obesity (FHO) was found in 77% of the obese subjects (one or both parents) as compared to FHO in 31% of the control subjects. Hypertension (HTN) was found in 18% of the obese subjects, whereas no control subject was found to be hypertensive. Similarly, among obese subjects, 7% were found to have type 2 diabetes (T2D) and 33.4% had impaired fasting glucose (IFG) in comparison to 2% of the control subjects were diabetic and 3% had IFG. Acanthosis nigricans was observed in 43% of the obese subjects as compared to 3.2% of the controls. Cardiovascular disease (CVD) was observed in 4.2% of the obese subjects, whereas no control subject had CVD. There was no significant difference found between body height (HT) of obese and control subjects. Waist and hip circumference and waist-to-hip-ratio (WHR) of obese subjects were found significantly greater than that of control subjects ($p < 0.05$). Obese subjects were found to have significantly higher SBP and DBP ($p < 0.05$) as

compared to the control subjects. Insulin levels and HOMA-IR values of obese subjects were found significantly ($p < 0.05$) higher as compared to that of control subjects. Leptin levels of the obese subjects were higher and LEPR levels were significantly lower as compared to that of control subjects. This above comparison of various parameters between obese and control subjects clearly indicates that obese individuals are more prone to develop various cardiometabolic disorders.

Genotypic frequencies of rs1137101 variant in obese and control subjects

The genotypes of rs1137101 variant were in Hardy–Weinberg equilibrium ($p > 0.05$) in both obese and control individuals. There was no significant difference ($p > 0.05$) in the genotype and allele frequencies of LEPR rs1137101 variant between obese and control subjects (Table 1). Analysis of whole data after stratification by sex revealed no significant difference in the genotype and allele frequencies ($p > 0.05$) between obese and non-obese males and similarly obese and non-obese females (Table 1). When all obese and control subjects were further stratified according to age in two categories ≤ 18 years and > 18 years, no significant difference was found in the genotype and allele frequencies between obese and control subjects > 18 years and ≤ 18 years ($p > 0.05$) (Table 1). Similarly, stratification of obese and control subjects > 18 years and ≤ 18 years into male and female subcategories revealed no significant difference in genotype and allele frequencies between > 18 years obese and control females, > 18 years obese and control males, ≤ 18 years obese and control females, and ≤ 18 years obese and control males (Table 1). Overall, these results revealed no significant difference in the frequencies of genotypes and alleles between obese and control subjects with and without stratification of the data according to age and sex.

Table 1: Genotype and allele distribution of LEPR rs1137101 variant in obese and non-obese subjects

	All subjects			Females			Males			> 18 years			Females > 18 years			Males > 18 years			≤ 18 years			Females ≤ 18 years			Males ≤ 18 years		
	Obese	Non-obese		Obese	Non-obese		Obese	Non-obese		Obese	Non-obese		Obese	Non-obese		Obese	Non-obese		Obese	Non-obese		Obese	Non-obese		Obese	Non-obese	
	n = 237	n = 131	X2/P/OR	n = 127	n = 74	X2/P/OR	n = 110	n = 57	X2/P/OR	n = 160	n = 87	X2/P/OR	n = 96	n = 41	X2/P/OR	n = 64	n = 46	X2/P/OR	n = 77	n = 44	X2/P/OR	n = 31	n = 33	X2/P/OR	n = 46	n = 11	X2/P/OR
Co-dominant model																											
GG	34 (14%)	18 (14%)		18 (14%)	13 (17%)		16 (14.5%)	6 (11%)		27 (17%)	12 (14%)		16 (17%)	6 (15%)		11 (17%)	6 (13%)		7 (9%)	7 (16%)		2 (6%)	7 (21%)		5 (11%)	0	
AG	98 (41%)	56 (43%)	0.073/0.964	56 (44%)	30 (41%)	0.48/0.78	42 (38%)	28 (49%)	1.93/0.38	70 (44%)	39 (45%)	0.41/0.81	43 (44%)	17 (41%)	0.35/0.83	27 (42%)	22 (48%)	0.50/0.77	28 (36%)	19 (43%)	2.51/0.28	13 (42%)	13 (39%)	3.02/0.21	15 (33%)	6 (55%)	2.55/0.27
AA	105 (44%)	57 (43%)		53 (41%)	31 (42%)		52 (47%)	23 (40%)		63 (39%)	36 (41%)		37 (39%)	18 (44%)		26 (40%)	18 (39%)		42 (55%)	18 (41%)		16 (52%)	13 (39%)		26 (56%)	5 (45%)	
Dominant model																											
GG+AG	132 (55%)	74 (56%)		74 (58%)	43 (58%)		58 (53%)	34 (60%)		97 (61%)	51 (59%)		59 (61%)	23 (56%)		38 (59%)	28 (61%)		35 (45%)	26 (59%)		15 (48%)	20 (61%)		20 (43%)	6 (55%)	
AA	105 (44%)	57 (43%)	0.02/0.88/0.9	53 (41%)	31 (42%)	0.41/0.52/1.0	52 (47%)	23 (40%)	0.72/0.39/0.7	63 (39%)	36 (41%)	0.09/0.7/0.5	37 (39%)	18 (44%)	0.34/0.55/1.2	26 (41%)	18 (39%)	0.02/0.87/0.9	42 (55%)	18 (41%)	2.08/0.14/0.5	16 (52%)	13 (39%)	0.96/0.32/0.5	26 (56%)	5 (45%)	0.43/0.50/0.6
Recessive model																											
GG	34 (14%)	18 (14%)		18 (14%)	13 (17%)		16 (14.5%)	6 (11%)		27 (17%)	12 (14%)		16 (17%)	6 (15%)		11 (17%)	6 (13%)		7 (9%)	7 (16%)		2 (6%)	7 (21%)		5 (11%)	0	
AG +AA	203 (85%)	113 (86%)	0.03/0.86/1.05	109 (85%)	61 (83%)	0.41/0.52/1.0	94 (85.4%)	51 (89%)	0.53/0.46/0.75	133 (83%)	75 (86%)	0.4/0.52/1.08	80 (83%)	35 (85%)	0.088/0.76/1.16	53 (83%)	40 (87%)	0.35/0.55/1.38	70 (90%)	37 (84%)	1.27/0.25/0.52	29 (94%)	26 (79%)	2.88/0.08/0.25	41 (89%)	11 (100%)	1.31/0.25
Alleles																											
G	166 (35%)	92 (35%)	0.001/0.97/0.99	92 (36%)	56 (38%)	0.10/0.74/0.77	74 (33.6%)	40 (35%)		124 (39%)	63 (36%)		75 (39%)	146 (73%)		49 (38%)	34 (37%)		42 (27%)	33 (38%)		17 (27%)	27 (41%)		25 (27%)	6 (27%)	
A	308 (65%)	170 (65%)		162 (64%)	92 (62%)		146 (66.3%)	74 (65%)	0.07/0.79/1.4	196 (61%)	111 (64%)	0.31/0.57/1.11	117 (61%)	53 (27%)	0.33/0.5/0.23	79 (62%)	58 (63%)	0.04/0.84/1.05	112 (73%)	55 (62%)	2.73/0.09/0.62	45 (72%)	39 (59%)	2.5/0.1/0.54	67 (73%)	16 (73%)	0/1/0.99

A = wild; G = polymorphic

LEPR rs1137101 variant and obesity

No significant association of any genotype or allele of the LEPR rs1137101 was observed with obesity ($p > 0.05$) in any of the genetic models (co-dominant, dominant or recessive models) used in the current study (Table 1). When the entire data were stratified according to age and sex, no significant association of any LEPR rs1137101 genotype or allele with obesity was found in any of the subcategories ($p > 0.05$). Thus, no age and sex-specific association of LEPR rs1137101 variant with obesity was observed in our study (Table 1).

LEPR rs1137101 variant and various obesity grades

The entire study population was stratified with respect to the WHO criteria of BMI into overweight (BMI 25.00–29.99), obese grade I (BMI 30.00–34.99), obese grade II (BMI 35.00–39.99), obese grade III (BMI ≥ 40.00), and super obese (BMI ≥ 50) groups. No significant association ($p > 0.05$) of any genotype (AA, AG or GG) with any grade of obesity was observed (Table 2).

Table 2: Genotype frequencies of LEPR rs1137101 variant across different obesity grades

	Obesity status						
	Obese			Non-obese			χ^2/p
Grades of obesity	GG	GA	AA	GG	GA	AA	
Grade I obese (BMI ≥ 30)	22%	42%	36%				2.07/0.355
Grade II obese (BMI ≥ 35)	13%	43%	44%	13%	46%	41%	0.17/0.91
Grade III obese (BMI ≥ 40)	15%	32%	53%				1.85/0.39

Chi-square analysis of genotypes.
BMI = body mass index.

LEPR rs1137101 variant, and anthropometric and metabolic traits

General linear model multivariate analysis revealed no significant association ($p > 0.05$) of the LEPR rs1137101 variant with any of the anthropometric and metabolic traits, including BW, HT, BMI, WC, HC, WHR, SBP, DBP, FBG, insulin levels, HOMA-IR, and leptin and LEPR levels (Table 3). There were no significant differences ($p > 0.05$) in anthropometric and metabolic parameters observed between carriers of AA, AG, and GG genotypes (Table 3).

Table 3: Differences of anthropometric and metabolic traits across genotypes of LEPR variant (mean ± SEM)

	AA n = 162	AG n = 156	GG n = 54	*p*-value
Body weight (kg)	82.42 ± 2.55	77.41 ± 3.47	73.4 ± 2	0.262
Height (m)	1.54 ± 0.016	1.59 ± 0.01	1.57 ± 0.01	0.217
BMI (kg/m^2)	33.69 ± 0.68	30 ± 1.06	29.38 ± 2	0.603
Waist(cm)	102.3 ± 1.71	92.3 ± 2.5	91.19 ± 1.77	0.622
Hip (cm)	111.8 ± 1.88	104 ± 2.18	102 ± 1.77	0.485
WHR	0.91 ± 0.007	0.87 ± 0.01	0.88 ± 0.006	0.925
SBP (mmHg)	121.45 ± 1.80	116 ± 1.8	115 ± 1.4	0.613
DBP (mmHg)	82 ± 1.18	78 ± 1.3	77.8 ± 0.95	0.436
FBS (mg/dL)	103.32 ± 3.98	93.1 ± 2	97.3 ± 2.64	0.536
Insulin (µIU/ml)	22.50 ± 1.93	15.7 ± 2.9	16.6 ± 1.37	0.841
HOMA	5.12 ± 0.51	3.5 ± 0.71	3.84 ± 0.36	0.887
Leptin (ng/ml)	28.78 ± 3.8	22.08 ± 1.838	24.28 ± 1.81	0.504
Leptin receptor (ng/ml)	15.53 ± 0.93	18.67 ± 1.65	17.3 ± 0.79	0.956

Data are presented as mean ± SEM and was compared by analysis of variance followed by Tukey Post hoc test. $p < 0.05$ was considered statistically significant.
BMI = body mass index; WHR = waist-to- hip ratio; SBP= systolic blood pressure; DBP = diastolic blood pressure; FBS = fasting blood sugar; HOMA = homeostasis model assessment.

DISCUSSION

The current study examined the association of the LEPR rs1137101 variant with obesity and associated anthropometric and metabolic parameters in obese and non-obese subjects of the Pakistani population. Both obese male and female subjects in the current study showed higher WHR than normal values, but male subjects with obesity had significantly higher WHR than female subjects with obesity, and therefore, they were predisposed to high risk of developing CVD (16). A WHR > 0.9 in males and > 0.85 in females is considered as a parameter for the diagnosis of CVD (17).

In the current study, obese subjects had significantly higher leptin levels as compared to control subjects; hyperleptinemia and leptin resistance might be involved in the development of hyperinsulinaemia and insulin resistance as shown by high HOMA-IR in the obese subjects of our study, a risk for development of T2D in later life (18). High FBG and high insulin levels found in obese subjects of our study might serve as a biomarker of disturbed carbohydrate metabolism and a future risk for T2D in these subjects. A significant correlation of plasma leptin levels with BMI in obese subjects was also seen in the current study. This observation is in agreement with previous reports (19, 20). Moreover, a positive correlation of leptin with BMI, WC, HC, HOMA-IR, and SBP in the obese subjects of the current study suggests that it is a possible risk factor for predisposition to HTN, CVD and T2D. A high leptin level induces endothelial damage and inflammatory reaction in blood vessels; these alterations may contribute to the pathogenesis of HTN (21).

Lack of association between rs1137101 variant of the LEPR gene and obesity in our study indicates that the amino acid change as a result of this polymorphism might not affect the phenotype of the subject in our population. This variant occurs in the CRH1 domain, which is not essential for high-affinity leptin binding (12). Stratigopoulos *et al.* observed no difference in adiposity between wild-type and Q223R isogenic mice fed a high fat diet (13). Recently, Verkerke *et al.* also reported that the rs1137101 variant in the LEPR extracellular CRH1 domain does not affect the rates at which leptin binds to and dissociates from its receptor (22).

Regarding the role of rs1137101 variant in obesity, the results of different association studies are controversial. The present study showed no association of this variant with obesity in adults or in the younger group (≤ 18 years). Likewise, no significant difference was observed in anthropometric parameters such as BMI, WC, HC, and WHR across genotypes of this variant. These findings are consistent with initial studies that also failed to identify any association of this variant with the risk of obesity (23–27). Later, the pooled and meta-analysis of genetic data by Heo *et al.* (10, 28) revealed conflicting results regarding the association of this polymorphism with BMI. Another meta-analysis by Paracchini *et al.* (29) involving 10 studies carried out in Asians, Caucasians, Pima Indians and Brazilians also reported no association of this variant with obesity. Large variation in the allelic frequency across different ethnicities and countries has been reported. Asian populations (Japan and Korea) showed relatively higher frequencies (0.85) of G allele than other populations. In contrast, our study reported a lower (0.35) allelic frequency of G allele. Yiannakouris *et al.* (30) observed an increased risk of obesity in G allele carriers, whereas Portolés *et al.* (31) found an inverse association of rs1137101 variant with obesity, which described that carriers of G allele had low BMI in the Mediterranean Spanish population; later on similar results were reported by Furusawa *et al.* (32) in a Pacific Island population demonstrating that carriers of A allele had high BW and BMI. Lack of association of rs1137101 genetic variant with obesity in the present study is in agreement with a recent study on Turkish children (33).

The present study also reported no difference in the metabolic parameters such as FBG, insulin, HOMA-IR, leptin and LEPR levels across genotypes of rs1137101 variant. Lack of association of rs1137101 variant with leptin levels in the current study is in line with a previous report (29). Guízar-Mendoza *et al.* (34) reported high body fat percentage and plasma leptin levels in carriers of A allele and low levels in G allele carriers in the Mexican adolescent population. However, another study reported high plasma leptin levels in G allele carriers in Greek subjects (30). There was no association of plasma LEPR levels with rs1137101 variant in our study that is similar to the observation of Ogawa *et al.* in the Japanese population (35).

The current study showed no association of rs1137101 variant with obesity in both males and females, whereas Masuo *et al.* reported gender-specific effect with association of this variant with obesity in Caucasian males (36). Another study in Caucasians reported an association of higher BMI with G allele carrier girls but not in boys (37). A recent meta-analysis revealed no association of rs1137101 variant with overweight and obesity including no gender-specific effect and hence supports the results of the current study (38). The lack of association of rs1137101 variant with obesity and obesity-related anthropometric and biochemical parameters seen in the current study is also in agreement with many previous reports (23–27, 29, 33, 37, 38). However, a recent study from Pakistan reported the association of rs1137101 variant with BMI, SBP, DBP and weight in obese subjects (39). This disagreement with our study may be attributable to the differences in the inclusion and exclusion criteria of the two studies.

In a nutshell, lack of association of rs1137101 variant of the LEPR gene with obesity and obesity-related anthropometric and metabolic traits in our study implies that this variant appears to have no role in causing susceptibility of our population to obesity and associated cardiometabolic disorders. The findings of the current study can be further validated by conducting similar studies with the same exclusion and inclusion criteria and by considering a bigger sample size.

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AUTHORS’ NOTE

The authors declare that they have no conflicts of interest.

REFERENCES

1. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R et al. Identification and expression cloning of a leptin receptor, OB-R. Cell 1995; **83:** 1263–71.
2. Jéquier E. Leptin signaling, adiposity, and energy balance. Ann N Y Acad Sci 2002; **967:** 379–88.
3. Winick JD, Stoffel M, Friedman JM. Identification of microsatellite markers linked to the human leptin receptor gene on chromosome 1. Genomics 1996; **36:** 221–2.
4. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI et al. Abnormal splicing of the leptin receptor in diabetic mice. Nature 1996; **379:** 632–5.
5. Huang L, Wang Z, Li C. Modulation of circulating leptin levels by its soluble receptor. J Biol Chem 2001; **276:** 6343–9.
6. Ge HF, Huang L, Pourbahram T, Li C. Generation of soluble leptin receptor by ectodomain shedding of membrane-spanning receptors in vitro and in vivo. J Biol Chem 2002; **277:** 45898–903.
7. Baumann H, Morella KK, White DW, Dembski M, Bailon PS et al. The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. Proc Natl Acad Sci USA 1996; **93:** 8374–8.
8. Bjørbaek C, Uotani S, da Silva B, Flier JS. Divergent signaling capabilities of the long and short isoforms of the leptin receptor. J Biol Chem 1997; **272:** 32686–95.
9. Zabeau L, Lavens D, Peelman F, Eyckerman S, Vandekerckhove J, Tavernier J. The ins and outs of leptin receptor activation. FEBS Lett 2003; **546:** 45–50.
10. Heo M, Leibel RL, Boyer BB, Chung WK, Koulu M, Karvonen MK et al. Pooling analysis of genetic data: the association of leptin receptor (LEPR) polymorphisms with variables related to human adiposity. Genetics 2001; **159:** 1163–78.
11. Thompson DB, Ravussin E, Bennett PH, Bogardus C. Structure and sequence variation at the human leptin receptor gene in lean and obese Pima Indians. Hum Mol Genet 1997; **6:** 675–9.
12. Peelman F, Beneden KV, Zabeau L, Iserentant H, Ulrichts P, Defeau D et al. Mapping of the leptin binding sites and design of a leptin antagonist. J Biol Chem 2004; **279:** 41038–46.
13. Stratigopoulos G, LeDuc CA, Matsuoka N, Gutman R, Rausch R, Robertson SA et al. Functional consequences of the human leptin receptor (LEPR) Q223R transversion. Obesity 2009; **17:** 126–35.
14. World Health Organization. Measuring obesity—classification and description of anthropometric data. In: Report on a WHO consultation on the epidemiology of obesity. 2nd ed. Warsaw, Poland: WHO; 1987: 2–7.
15. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; **28:** 412–9.
16. Sundell J, Raitakari OT, Viikari J, Kantola I, Nuutila P, Knuuti J. Both BMI and waist circumference are associated with coronary vasoreactivity in overweight and obese men. Obes Facts 2012; **5:** 693–9.
17. Dalton M, Cameron AJ, Zimmet PZ, Shaw JE, Jolley D, Dunstan DW et al. AusDiab Steering Committee. Waist circumference, waist-hip ratio and body mass index and their correlation with cardiovascular disease risk factors in Australian adults. J Intern Med 2003; **254:** 555–63.
18. Covey SD, Wideman RD, McDonald C, Unniappan S, Huynh F, Asadi A et al. The pancreatic beta cell is a key site for mediating the effects of leptin on glucose homeostasis. Cell Metab 2006; **4:** 291–302.
19. Kostalova L, Leskova L, Kapellerova A, Strbak V. Body mass, plasma leptin, glucose insulin and C-peptide in offspring of diabetic and non-diabetic mothers. Eur J Endocrinol 2001; **145:** 53–8.
20. Shahid A, Lone KP, Saeed S, Arslan M. Male offspring of both diabetic parents have higher Insulin resistance and serum leptin levels compared to those with one diabetic parent. Hormones 2008; **7:** 313–9.
21. Correia ML, Haynes WG. Leptin, obesity and cardiovascular disease. Curr Opin Nephrol Hypertens. 2004; **13:** 215–23.
22. Verkerke H, Naylor C, Zabeau L, Tavernier J, Petri WA Jr, Marie C. Kinetics of leptin binding to the Q223R leptin receptor. PLoS One 2014; **9:** e94843.
23. Considine RV, Considine EL, Williams CJ, Hyde TM, Caro JF. The hypothalamic leptin receptor in humans: identification of incidental sequence polymorphisms and absence of the db/db mouse and fa/fa rat mutations. Diabetes. 1996; **45:** 992–4.
24. Echwald SM, Sørensen TD, Sørensen TI, Tybjaerg-Hansen A, Andersen T, Chung WK et al. Amino acid variants in the human leptin receptor: lack of association to juvenile onset obesity. Biochem Biophys Res Commun 1997; **233:** 248–52.
25. Gotoda T, Manning BS, Goldstone AP, Imrie H, Evans AL, Strosberg AD et al. Leptin receptor gene variation and obesity: lack of association in a white British male population. Hum Mol Genet 1997; **6**: 869–76.
26. Silver K, Walston J, Chung WK, Yao F, Parikh VV, Andersen R et al. The Gln223Arg and Lys656Asn polymorphisms in the human leptin receptor do not associate with traits related to obesity. Diabetes 1997; **46:** 1898–900.
27. Matsuoka N, Ogawa Y, Hosoda K, Matsuda J, Masuzaki H, Miyawaki T et al. Human leptin receptor gene in obese Japanese subjects: evidence against either obesity-causing mutations or association of sequence variants with obesity. Diabetologia 1997; **40:** 1204–10.
28. Heo M, Leibel RL, Fontaine KR, Boyer BB, Chung WK, Koulu M et al. A meta-analytic investigation of linkage and association of common leptin receptor (LEPR) polymorphisms with body mass index and waist circumference. Int J Obes Relat Metab Disord 2002; **26:** 640–6.
29. Paracchini V, Pedotti P, Taioli E. Genetics of leptin and obesity: a HuGE review. Am J Epidemiol 2005; **162:** 101–14.
30. Yiannakouris N, Yannakoulia M, Melistas L, Chan JL, Klimis-Zacas D, Mantzoros CS. The Q223R polymorphism of the leptin receptor gene is significantly associated with obesity and predicts a small percentage of body weight and body composition variability. J Clin Endocrinol Metabol 2001; **86:** 4434–9.
31. Portolés O, Sorlí JV, Francés F, Coltell O, González JI, Sáiz C et al. Effect of genetic variation in the leptin gene promoter and the leptin receptor gene on obesity risk in a population-based case-control study in Spain. Eur J Epidemiol 2006; **21:** 605–12.
32. Furusawa T1, Naka I, Yamauchi T, Natsuhara K, Kimura R, Nakazawa M et al. The Q223R polymorphism in LEPR is associated with obesity in Pacific Islanders. Hum Genet 2010; **127:** 287–94.
33. Komşu-Ornek Z, Demirel F, Dursun A, Ermiş B, Pişkin E, Bideci A. Leptin receptor gene Gln223Arg polymorphism is not associated with obesity and metabolic syndrome in Turkish children. Turk J Pediatr 2012; **54:** 20–4.
34. Guízar-Mendoza JM, Amador-Licona N, Flores-Martínez SE, López-Cardona MG, Ahuatzin-Trémary R, Sánchez-Corona J. Association analysis of the Gln223Arg polymorphism in the human leptin receptor gene, and traits related to obesity in Mexican adolescents. J Hum Hypertens 2005; **19:** 341–6.
35. Ogawa T, Hirose H, Yamamoto Y, Nishikai K, Miyashita K, Nakamura H et al. Relationships between serum soluble leptin receptor level and serum leptin and adiponectin levels, insulin resistance index, lipid profile, and leptin receptor gene polymorphisms in the Japanese population. Metabolism 2004; **53:** 879–85.
36. Masuo K, Straznicky NE, Lambert GW, Katsuya T, Sugimoto K, Rakugi H et al. Leptin-receptor polymorphisms relate to obesity through blunted leptin mediated sympathetic nerve activation in a Caucasian male population. Hypertens Res 2008; **31:**1093–100.
37. Riestra P, Garcia-Anguita A, Schoppen S, Lopez-Simon L, De Oya M, Garcés C. Sex-specific association between leptin receptor polymorphisms and leptin levels and BMI in healthy adolescents. Acta Paediatr 2010; **99:** 1527–30.
38. Bender N, Allemann N, Marek D, Vollenweider P, Waeber G, Mooser V et al. Association between variants of the leptin receptor gene (LEPR) and overweight: a systematic review and an analysis of the CoLaus study. PLoS One 2011; **6:** e26157.
39. Shabana NA, Hasnain S. Association of the leptin receptor Gln223 Arg polymorphism with lipid profile in obese Pakistani subjects. Nutrition 2015; **31:** 1136.

ORIGINAL ARTICLE

The Effect of Obesity and Body Mass Index on Post-transplant Renal Graft Function

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ABSTRACT

Objective: *The aim of this five-year study was to investigate the influence of body mass index (BMI) and obesity on post-transplant renal graft function.*
Methods: *This research monitored 500 patients of both genders who had received kidney transplants. Post-transplant measurements of the recipients' biochemical parameters, anthropometric parameters and renal function were performed. Two methods were also applied for assessing renal function: (a) the Cockcroft–Gault formula and (b) the serum creatinine clearance.*
Results: *Five years after transplantation, the patients showed an increase in obesity, accompanied by a lower glomerular filtration rate (GFR). Although the Cockcroft–Gault values were mild, the GFR percentage tended to decrease from the first to the fifth year, with higher serum creatinine levels.*
Conclusion: *The results reflected an increase in body weight and BMI in the five years after transplantation. This led to a lower GFR and subsequent graft damage during this time period.*

Keywords: Anthropometry, glomerular function, hyperfiltration, obesity, renal transplantation

INTRODUCTION

Obesity is a serious risk factor for kidney deterioration and failure. This is true for healthy people as well as those with chronic kidney disease (CKD) (1). The exact mechanisms whereby obesity may worsen or cause CKD remain unclear; however, it is believed that kidney damage is produced by haemodynamic and hormonal effects (2). After a renal graft, it is fairly common for patients to gain weight (3). This is largely due to their improved appetite, the disappearance of dietary restrictions stemming from haemodialysis and CKD, as well as the use of immunosuppressive medications and steroids (4). A wide range of studies have found that kidney graft recipients gain an average of 5–10 kg, which is directly linked to a lower patient survival rate as well as graft loss (5–7). A post-transplant weight gain contributes to an adverse cardiovascular risk profile and can play a role in the pathogenesis of renal graft dysfunction. In fact, it has been associated with post-transplant hypertension, diabetes, dyslipidaemia and ischaemic heart disease (8). Many studies have focussed on the short-term effects of the renal graft deterioration due to obesity and factors related to the post-transplant weight gain. However, there are no long-term data regarding the impact of this weight gain on renal graft function (9–12). Therefore, the aim of this research was to analyse the influence of the body mass index (BMI) and obesity on renal graft function in the five-year period after the transplant. For this purpose, the glomerular filtration rate (GFR) was calculated by two different methods: (a) the Cockcroft–Gault formula (CCr) and (b) the serum creatinine clearance (SCrCl).

SUBJECTS AND METHODS

The population sample consisted of 500 patients (278 men and 222 women), 16–80 years of age. All of the subjects had received renal grafts, and periodically visited the kidney transplant unit at the Virgen de las Nieves University Hospital in Granada, Spain. Patients were chosen because their visits to the hospital for post transplant follow-up and monitoring took place within the time period of our research (June 2010-15). Figure 1 shows their underlying disease.

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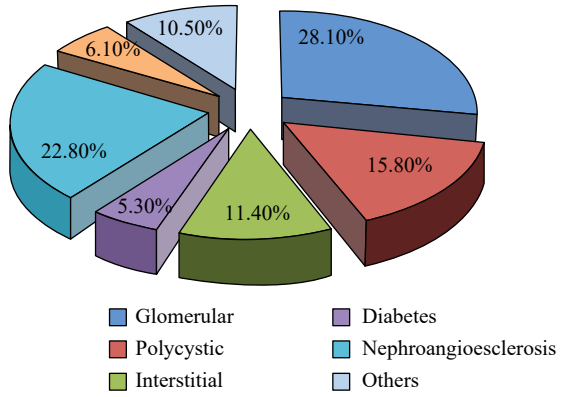

Figure 1: Causes of chronic kidney disease.

The participants' weight and height were measured with a Perperson 113481 scale stadiometer. Their BMI was then calculated as their body weight in kilograms divided by the square of their height in centimetres (weight/height^2). The resulting BMI was evaluated based on the World Health Organization (WHO) classification: (a) underweight (BMI < 18.5 kg/m^2); (b) normal weight (BMI = 18.5–24.99 kg/m^2); (c) overweight (BMI = 25–29.99 kg/m^2); (d) obese (BMI ≥ 30 kg/m^2). Renal function was assessed with the Cockcroft–Gault (CCr) formula and the serum creatinine clearance (SCrCl).

Accordingly the Cockcroft–Gault formula determined the GFR as follows:

In the case of males, normal values are 70 ± 14 mL/min/m^2, whereas for females, they are 60 ± 10 mL/min/m^2. Based on the decreasing GFR, values were classified as follows: (a) Mild decrease (60–41 mL/min); (b) Moderate decrease (40–21 mL/min); (c) Severe decrease (< 21 mL/min).

Serum creatinine clearance determines serum creatinine (SCr) levels in the blood by means of the kinetic Jaffé reaction, in which the creatinine reacts with alkaline picrate to produce a red-coloured complex. Serum creatinine levels were calculated by measuring an increase in absorbance at 512 nm. The rate of the dye formation (colour intensity) is directly proportional to the creatinine concentration in the specimen. A normal result is 0.7–1.3 mg/dL for males and 0.6–1.1 mg/dL for females, respectively. Generally speaking, normal values range from 0.8 to 1.4 mg/dL.

Before being discharged from the hospital, all of the participants were advised to consume 1.4–1.5 g/kg/day of diet protein (30–35 kcal/kg/day) during the first three months after the renal graft. Moreover, they were told that lipids should not exceed 30% of the total dietary intake and simple sugars should be avoided. After this three-month period, patients could reduce their protein consumption to 1 g/kg/day.

The GFR indicates stages of CKD as specified in the guidelines of the National Kidney Foundation (Table 1).

Table 1: Classification of the stages of chronic kidney disease in the guidelines of the National Kidney Foundation

Stage	Description	GFR (mL/min/1.73 m^2)
1	Kidney damage with a normal or higher GFR	≥ 90
2	Kidney damage with a slightly lower GFR	60–89
3	Mild-to-moderate decrease in GFR	30–59
4	Significant decrease in GFR	15–29
5	Kidney failure or dialysis	< 15

GFR = glomerular filtration rate.

Statistical analysis

The statistical analysis was performed with the software package IBM SPSS Statistics 20. Analysis of variance (ANOVA) was used to analyse the differences between the BMI and renal function. All data were expressed as frequencies, percentages and mean values + standard deviation. Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

As can be observed in Table 2, the percentage of overweight patients remained more or less constant in the five-year period after the kidney transplant. However, there was an increase in the patients with obesity, whereas each year the number of normal-weight and underweight patients became steadily lower. In regard to renal function, Table 3 shows that even though Cockcroft–Gault values reflect a mild decrease, this percentage became progressively lower during the five-year time period. The same occurred with the SCr levels in the blood, where from the first to the fifth year, the values tended to increase until they exceeded our laboratory reference values (normal values of 0.8–1.4 mg/dL).

Table 2: Percentage of patients classified according to the WHO classification

BMI (kg/m^2)	Post-transplant years				
	1st*	2nd*	3rd*	4th*	5th*
Underweight: < 18.5	6.1%	5.9%	6.3%	5.3%	5.6%
Normal weight: 18.5–24.99	33.1%	32.5%	31.5%	32.6%	29.1%
Overweight: 25–29.99	34.2%	33.1%	33%	32.6%	33.6%
Obesity: ≥ 30	26.6%	28.5%	29.3%	29.6%	31.8%

*$p < 0.05$.

Table 3: Evolution of the renal function in five years by two methods: the Cockcroft–Gault formula (CCr; mL/min) and serum creatinine (SCr; mg/dL)

Filtration/year	Mean	SD
CCr 1st year	62.09	22.90
CCr 2nd year	61.61	24.36
CCr 3rd year	60.76	24.96
CCr 4th year	60.49	26.00
CCr 5th year	60.15	26.11
SCr 1st year	1.68	0.99
SCr 2nd year	1.77	1.33
SCr 3th year	1.80	1.35
SCr 4th year	1.79	1.20
SCr 5th year	1.78	1.15

*$p < 0.05$.
CCr = Cockcroft–Gault formula; SCr = serum creatinine; SD = standard deviation.

Even though creatinine levels remained elevated, when they are correlated with the BMI, they show a statistically significant increase with a higher BMI, especially in the first three years. However, the tendency was for creatinine levels to rise each year and remain high, especially in patients who are overweight and obese (Table 4).

Table 4: Five-year serum creatinine levels and the BMI (the WHO classification)

GFR	BMI (kg/m²)	N	Mean	SD
SCr 1st year*	Underweight: < 18.5	25	1.31	0.45
	Normal weight: 18.5–24.99	130	1.56	0.66
	Overweight: 25–29.99	175	1.59	0.53
	Obesity: ≥ 30	170	1.68	0.51
SCr 2nd year*	Underweight: < 18.5	25	1.30	0.39
	Normal weight: 18.5–24.99	135	1.55	0.65
	Overweight: 25–29.99	172	1.64	0.90
	Obesity: ≥ 30	168	1.72	0.55
SCr 3rd year*	Underweight: < 18.5	25	1.37	0.43
	Normal weight: 18.5–24.99	135	1.55	0.63
	Overweight: 25–29.99	172	1.65	0.73
	Obesity: ≥ 30	168	1.70	0.57
SCr 4th year	Underweight: < 18.5	25	1.49	0.73
	Normal weight: 18.5–24.99	137	1.56	0.71
	Overweight: 25–29.99	170	1.69	0.83
	Obesity: ≥ 30	168	1.73	0.63
SCr 5th year	Underweight: < 18.5	25	1.70	1.38
	Normal weight: 18.5–24.99	137	1.65	0.94
	Overweight: 25–29.99	170	1.80	1.26
	Obesity: ≥ 30	168	1.79	0.73

*$p < 0.05$.
BMI = body mass index; GFR = glomerular filtration rate; SCr = serum creatinine; SD = standard deviation.

Regarding the glomerular function (Table 5), instead of a decrease, the results obtained with the Cockcroft–Gault formula show a statistically significant increase when correlated with the BMI. During the five-year period after the transplant, the values generally reflect a mild-to-moderate loss decrease (60–41 mL/min).

Table 5: Evolution of the Cockcroft–Gault filtration (CCr) for years and the BMI (the WHO classification)

GFR	BMI (kg/m2)	N	Media	SD
CCr 1st year*	Underweight: < 18.5	25	55.21	16.46
	Normal weight: 18.5–24.99	130	58.37	21.35
	Overweight: 25–29.99	175	63	22.18
	Obesity: ≥ 30	170	67.82	22.79
CCr 2nd year*	Underweight: < 18.5	25	53.58	14.42
	Normal weight: 18.5–24.99	135	58.94	23.17
	Overweight: 25–29.99	172	63.33	21.93
	Obesity: ≥ 30	168	66.55	23.44
CCr 3rd year*	Underweight: < 18.5	25	51.56	13.20
	Normal weight: 18.5–24.99	135	57.87	22.57
	Overweight: 25–29.99	172	62.28	22.21
	Obesity: ≥ 30	168	68.04	26.09
CCr 4th year*	Underweight: < 18.5	25	48.99	14.44
	Normal weight: 18.5–24.99	137	58.12	23.30
	Overweight: 25–29.99	170	61.63	23.73
	Obesity: ≥ 30	168	68.63	28.25
CCr 5th year*	Underweight: < 18.5	25	48.66	18.78
	Normal weight: 18.5–24.99	137	56.56	25.11
	Overweight: 25–29.99	170	61.77	25.27
	Obesity: ≥ 30	168	66.65	28.79

*$p < 0.05$.
BMI = body mass index; GFR = glomerular filtration rate; CCr = Cockcroft–Gault filtration; SD = standard deviation.

DISCUSSION

The results of our study found that there was a high prevalence of obese and overweight patients, which steadily increased over the years. Many research studies have obtained a significant amount of experimental and clinical data that highlight the fact that obesity is an important risk factor for kidney disease (13, 14). Nevertheless, there is a scarcity of epidemiological data that directly point to obesity as a trigger or direct cause of kidney disease. Certain studies have focussed on the association between obesity and proteinuria in the general population (15, 16). However, very few epidemiological studies have quantified the possible relation between obesity and kidney failure (17, 18).

The prevalence of obesity throughout the world is soaring. As a result, there are a growing number of patients with cardiovascular disease, metabolic syndrome and CKD (19). As specified in the Kidney

Disease: Improving Global Outcomes (KDIGO) guidelines, there is an association between obesity, renal graft dysfunction, graft loss and mortality in the kidney transplant recipients. There is also a higher risk of perioperative complications, illnesses and concomitants (20, 21).

In this research, we assessed renal function in a sample of kidney graft recipients during the five-year post-transplant period with a particular focus on their BMI. Many studies have shown how weight gain after transplantation can have long-term adverse effects on the patients' renal function. However, in these studies, weight gain was mainly due to the metabolic syndrome, including hypertension, diabetes and hyperlipidaemia (22). Our study demonstrated that the weight gain in the critical five years after the transplant has an extremely negative impact on kidney function, which can be explained by the increased metabolic load stemming from the weight gain (23).

Factors that explain long-term weight gain in transplant recipients include their improved appetite, disappearance of dietary restrictions and limitations on physical activity due to immunosuppression (24). In regard to delayed graft function, both obesity and overweight prevent the kidney from maintaining a satisfactory filtration rate. This agrees with other studies that underline obesity as a risk factor that endangers normal kidney function (25).

Our research showed that a BMI > 25 kg/m^2 after the kidney transplant correlated with a higher percentage of graft dysfunction. Moreover, as the patients' BMI increased, there was a lower GFR, as measured by the CCr and the SCrCl methods. Since the SCrCl is a kidney damage marker, as the BMI increased, there was a greater kidney distress. This coincides with other studies that found that a higher BMI (26–28) was accompanied by higher creatinine levels, which signify a decrease in glomerular filtration (29, 30).

CONCLUSION

The results showed that five years after kidney transplantation, recipients experienced a weight gain and had a higher BMI. This factor contributed to graft dysfunction as reflected in a lower GFR and graft complications due to the kidney damage and a progressive loss of kidney function.

AUTHORS' NOTE

MS Adelina conceived paper, oversaw data collection, conducted data analysis, wrote manuscript and approved final version. NN Ana María participated in the study design, data analysis and interpretation, critically revised manuscript and approved final version. I García participated in the study design, data analysis, interpretation of data, revision of manuscript and approved final version. RF Castillo participated in the study design, interpretation of data, revision of manuscript and approved final version.

REFERENCES

1. Foster MC, Hwang SJ, Larson MG, Lichtman JH, Parikh NI, Vasan RS et al. Overweight, obesity, and the development of stage 3 CKD: the Framingham Heart Study. Am J Kidney Dis 2008; **52:** 39–48.
2. Hsu CY, McCulloch CE, Iribarren C, Darbinian J, Go AS. Body mass index and risk for end-stage renal disease. Ann Intern Med 2006; **144**: 21–8.
3. Praga M. Obesity: a neglected culprit in renal disease. Nephrol Dial Transplant 2002; **17:** 1157e9.
4. Elagroudy AE, Wafa EW, Gheith OE, Shehab el-Dein AB, Ghoneim MA. Weight gain after renal transplantation is a risk factor for patient and graft outcome. Transplantation 2004; **77:** 1381–5.
5. Clunk JM, Lin CY, Curtis JJ. Variables affecting weight gain in renal transplant recipients. Am J Kidney Dis 2001; **38:** 349–53.
6. Cashion AK, Sanchez ZV, Cowan PA, Hathaway DK, Lo Costello A, Gaber AO. Changes in weight during the first year after kidney transplantation. Prog Transplant 2007; **17:** 40–7.
7. Elster EA, Leeser DB, Morrissette C, Pepek JM, Quiko A, Hale DA et al. Obesity following kidney transplantation and steroid avoidance immunosuppression. Clin Transplant 2008; **22**: 354–9.
8. Armstrong KA, Campbell SB, Hawley CM, Nicol DL, Johnson DW, Isbel NM. Obesity is associated with worsening cardiovascular risk factor profiles and proteinuria progression in renal transplant recipients. Am J Transplant 2005; **5:** 2710–18.
9. Johnson CP, Gallagher-Lepak S, Zhu YR, Porth C, Kelber S, Roza AM et al. Factors influencing weight gain after renal transplantation. Transplantation 1993; **56:** 822–7.
10. Nazemian F, Naghibi M. Weight-gain-related factors in renal transplantation. Exp Clin Transplant 2005; **3:** 329–32.
11. Cashion AK, Hathaway DK, Stanfill A, Thomas F, Ziebarth JD, Cui Y et al. Pre-transplant predictors of one-year weight gain after kidney transplantation. Clin Transplant 2014; **28:** 1271–8.
12. Ryan KJ, Casas JM, Mash LE, McLellan SL, Lloyd LE, Stinear JW et al. The effect of intensive nutrition interventions on weight gain after kidney transplantation: protocol of a randomised controlled trial. BMC Nephrol 2014; **15:** 148.
13. Zrim S, Furlong T, Grace B, Meade A. Body mass index and postoperative complications in kidney transplant recipients. Nephrology 2012; **17:** 582–7.
14. Aalten J, Christiaans M, de Fijter H, Hené R, van der Heijde JH, Roodnat J et al. The influence of obesity on short and long-term graft and patient survival after renal transplantation. Transpl Int 2006; **19:** 901–7.
15. Shoham DA, Durazo-Arvizu R, Kramer H, Luke A, Vupputuri S, Kshirsagar A et al. Sugary soda consumption and albuminuria: results from the National Health and Nutrition Examination Survey, 1999–2004. PLoS One 2008; **3:** e3431.
16. Palaniappan L, Carnethon M, Fortmann SP. Association between microalbuminuria and the metabolic syndrome: NHANES III. Am J Hypertens 2003; **16:** 952–8.
17. Chang S, Coates O, McDonald S. Effects of body mass index at transplant on outcomes of kidney transplantation. Transplantation 2007; **84:** 981.
18. Huang E, Bunnapradist S. Pre-transplant weight loss and survival after kidney transplantation. Am J Nephrol 2015; **41:** 448–55.

19. Fernández Castillo R, Fernández Gallegos R, Peña Amaro MP, Esteban de la Rosa RJ. Assessment of lipid profiles and bone mineral density in renal transplant patients. Nutr Hosp 2015; **31:** 2503–10.
20. Chang A, Greene TH, Wang X, Kendrick C, Kramer H, Wright J et al. The effects of weight change on glomerular filtration rate. Nephrol Dial Transplant 2015; **30:** 1870–7.
21. Booth JC, Joseph JT, Jindal RM. Influence of hipercolesterolemia on patient and graft survival in recipients of kidney transplants. Clin Transplant 2003; **17:** 101–5.
22. Chan G, Garneau P, Hajjar R. The impact and treatment of obesity in kidney transplant candidates and recipients. Can J Kidney Health Dis 2015; **2:** 26.
23. Boratynska M, Banasik M, Watorek E, Klinger M, Dorobisz A, Szyber P. Influence of hipercolesterolemia and acute graft rejection on chronic nephropathy development in renal transplant recipient. Transplant Proc 2003; **35:** 2209–12.
24. Suarez O, Pardo M, Gonzalez S, Escobar-Serna DP, Castaneda DA, Rodriguez D et al. Diabetes mellitus and renal transplantation in adults: is there enough evidence for diagnosis, treatment, and prevention of new-onset diabetes after renal transplantation? Transplant Proc 2014; **46:** 3015–20.
25. Gourishankar S, Grebe SO, Mueller TF. Prediction of kidney graft failure using clinical scoring tools. Clin Transplant 2013; **27:** 517–22.
26. Hricik DE. Metabolic syndrome in kidney transplantation: management of risk factors. Clin J Am Soc Nephrol 2011; **6:** 1781–5.
27. Hartmann EL, Kitzman D, Rocco M, Leng X, Klepin H, Gordon M et al. Physical function in older candidates for renal transplantation: an impaired population. Clin J Am Soc Nephrol 2009; **4:** 588–94.
28. Ditonno P, Lucarelli G, Impedovo SV, Spilotros M, Grandaliano G, Selvaggi FP et al. Obesity in kidney transplantation affects renal function but not graft and patient survival. Transplant Proc 2011; **43:** 367–72.
29. Grosso G, Corona D, Mistretta A, Zerbo D, Sinagra N, Giaquinta A et al. The role of obesity in kidney transplantation outcome. Transplant Proc 2012; **44:** 1864–8.
30. Ravindra K, Irish W, Vikraman D, Castleberry A, Barbas A, Sanoff S et al. Impact of recipient BMI: implications for ECD renal transplantation. Am J Transplant 2013; **13:** 195.

ORIGINAL ARTICLE

CA 19-9 Could Predict Both Advanced Disease and Nodal Involvement in Primary Mucinous Endometrial Cancers*

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ABSTRACT

Objective: *As mucinous endometrial carcinoma is a rare disease, a study to evaluate and find predictive factors of mucinous endometrial carcinoma for advanced disease and nodal involvement was performed.*

Methods: *Patients who were operated on at the Gynaecologic Oncology Department of Zekai Tahir Burak Women's Health, Education and Research Hospital between January 2006 and January 2013 were evaluated, and patients with primary mucinous endometrial carcinoma were retrospectively analysed.*

Results: *A total of 16 primary mucinous endometrial carcinoma patients were detected. Patients were evaluated based on two groups, according to stage. Group 1 consisted of stage IA patients and group 2 consisted of patients with higher stages than 1A (advanced disease). Twelve patients (75%) were stage IA whereas four patients (25%) had higher than stage IA disease. Lymph node metastasis and lymphovascular space invasion were significantly positive in advanced disease (*p *= 0.011 and* p *= 0.01, respectively). CA 125 and CA 19-9 levels were also significantly elevated in patients with advanced disease (*p *= 0.021 and* p *= 0.039). On the other hand, only CA 19-9 was significantly elevated in the patients with nodal metastasis.*

Conclusion: *Pre-operative work-up of CA-19.9 could predict both advanced disease and lymph node metastasis for mucinous endometrial cancers.*

Keywords: Endometrial cancer, lymph node, lymphovascular space invasion, mucinous tumour, tumour markers.

INTRODUCTION

Endometrial cancer is the most common gynaecologic malignancy. Endometrioid histology is the main type of this carcinoma and is associated with a good prognosis and long survival. Endometrial cancer is generally detected in the early stages, without any nodal involvement and metastasis. Despite the rarity of the non-endometrioid type, this type behaves aggressively and constitutes the major part of recurrences and deaths. Extrauterine disease is commonly detected with the non-endometrioid type and nodal involvement may also exist (1, 2). Mucinous endometrial carcinoma is one of the non-endometrioid carcinomas and is less than 10% of all endometrial cancers (3).

Tumour stage, grade, histologic type and depth of myometrial invasion are important prognostic factors of endometrial cancer (4). Lymph node metastasis could upstage a patient and has a direct role in survival. It has been previously mentioned that mucinous histology is also a risk factor for lymph node metastasis (5). Moreover, tumour markers are generally evaluated in gynaecologic oncology practice, and have a role in the management and follow-up (6). In that clinical setting, a

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study to evaluate primary mucinous endometrial cancers was performed within clinicopathologic parameters.

SUBJECTS AND METHODS

This institutional, ethical board-approved study was performed at Zekai Tahir Burak Women's Health, Education and Research Hospital, Turkey. Patients who were operated at the Gynaecologic Oncology Department between January 2006 and January 2013 were evaluated, and patients with primary mucinous endometrial carcinoma were retrospectively analysed.

Mucinous endometrial carcinoma was defined if the intracytoplasmic mucin collection is more than 50% of the total tumour cell population. These cells are positive for mucicarmine and periodic acid-Schiff stain (7, 8). We excluded cases with less than 50% of mucinous architecture. Patient's age, menopausal status, parity, symptom, body mass index (BMI), stage, grade, myometrial invasion, cervical involvement, tumour diameter, lymphovascular space invasion (LVSI), lymph node count-metastasis, peritoneal cytology, cancer antigen (CA) 125, and CA 19-9 values were considered for analysis.

All patients underwent hysterectomy, bilateral salpingo-oophorectomy, pelvic-paraaortic lymphadenectomy and omentectomy. The International Federation of Gynaecology and Obstetrics (FIGO) staging system for endometrial carcinoma (2009) was performed for clinical staging. All the operations were performed by certified gynaecological oncologists and pathology specimens were interpreted by senior pathologists in gynaecological oncology.

The statistical analyses were performed by using the Statistics Package for the Social Sciences version 17.0 (SPSS, Chicago, IL, USA). The Chi-square and Fisher's exact tests were used to analyse nominal variables in the form of frequency tables. Non-nominally distributed metric variables were analysed by the Mann–Whitney *U* test. *p*-values < 0.05 were considered statistically significant. Values were expressed as mean ± SD, unless stated otherwise.

RESULTS

A total of 16 primary mucinous endometrial carcinoma patients were detected. The incidence of mucinous endometrial cancer out of 658 endometrial carcinoma patients is 2.43%. The median age of the patients was 60, and 12 (75%) were post-menopausal. Vaginal bleeding (n = 12, 75%) was the main symptom and pelvic pain was the most common accompanying symptom. Patients were evaluated based on two groups, according to the stage. Group 1 consisted of stage IA patients, and group 2 consisted of patients with higher stages than 1A (advanced disease). Twelve patients (75%) were stage IA, four patients (25%) had higher than stage IA disease; two patients (12.5%) were stage IB, and two patients (12.5%) were stage IIIC (2). Mean age, BMI, parity, tumour diameter, lymph node count, and menopausal status were not significantly different between the groups (Table 1). Lymph node metastasis and LVSI were significantly positive in advanced disease ($p = 0.011$ and $p = 0.01$, respectively). CA 125 and CA 19-9 levels were also significantly elevated in patients with advanced disease ($p = 0.021$ and $p = 0.039$) (Table 1).

Table 1: Clinicopathological characteristics of patients according to stage

Characteristics	Stage IA (n = 12)	> Stage IA (n = 4)	*p*-value
Age	57.1 ± 5.4	58.7 ± 11.7	> 0.05
BMI	31.3 ± 4.6	32.4 ± 5.7	> 0.05
Parity	3.3 ± 1.8	4.1 ± 2.3	> 0.05
Postmenopausal	9 (75%)	3 (75%)	> 0.05
Tumour diameter	1.4 ± 0.7	2.1 ± 1.5	> 0.05
Pelvic lymph node count	47.5 ± 23.1	44.0 ± 15.7	> 0.05
Paraaortic lymph node count	19.0 ± 10.1	17.7 ± 3.5	> 0.05
Lymph node metastasis	0 (0%)	2 (50%)	0.011
LVSI	1 (8.3%)	3 (75%)	0.01
CA-125	17.4 ± 13.5	48.5 ± 31.4	0.021
CA-19.9	19.0 ± 11.3	88.2 ± 76.3	0.039

BMI = body mass index; LVSI = lymphovascular space invasion.

Lymph node metastasis was detected in two patients (12.5%). One of these patients had isolated paraaortic lymph node metastasis and the other one had pelvic and para-aortic lymph node metastasis. Only CA 19-9 was significantly elevated in the patients with nodal metastasis ($p = 0.026$). The other parameters were similar and not significant (Table 2).

Table 2: Tumour marker levels of patients according to lymph node metastasis

Characteristics	Negative lymph node metastasis (n = 14)	Positive lymph node metastasis (n = 2)	*p*
CA-125	20.2 ± 15.3	50.9 ± 40.3	0.08
CA-19.9	18.6 ± 12.1	148.5 ± 51.6	0.026

DISCUSSION

Mucinous metaplasia of the endometrial glands showed variable degrees of epithelial change with or without intraglandular micropapillary features. Additionally, a complex, glandular architecture similar to low-grade,

mucinous adenocarcinoma could also be seen (9). Lesions with these histopathologic features could be precancerous lesions of mucinous adenocarcinoma (10). Cytologic atypia is an important predictor of malignancy risk for mucinous proliferations (11). In that clinical setting, care should be taken during the evaluation of these patients.

Mucinous endometrial carcinoma is a rare histologic subtype of endometrial cancer. Because of the limited number of studies, only retrospective, small cohorts shape the clinical management. Stage and grade are the most important prognostic factors. Mucinous tumours generally present in the elderly people. Mucinous endometrial carcinomas may present as an advanced stage tumour (2). However, Jalloul *et al*. (12) had reported mucinous endometrial carcinomas as low-grade and early-stage tumours. Nevertheless, the frequency of deep myometrial invasion should not be underestimated for mucinous endometrial cancers (13). Within this study, 12 patients (75%) were stage IA and 10 patients (62.5%) were in grade 1 histology.

Musa *et al*. (5) performed a case-control study with mucinous and endometrioid endometrial carcinomas and found mucinous histology as an independent predictor of lymph node metastasis. However, the risk of deep myometrial invasion does not increase. A recent analysis of surveillance, epidemiology, and end results (SEER) analysed 103 097 endometrioid endometrial adenocarcinoma patients (98.5%), and 1562 mucinous endometrial adenocarcinoma patients (1.5%), and found the extent of nodal metastasis higher for patients with a mucinous carcinoma (3). Despite these findings, the survival period for mucinous endometrial carcinoma patients does not decrease after stage-matched analysis (2). The SEER database also showed that mucinous histology does not affect cancer-specific survival (3).

Disease-specific survival for mucinous endometrial carcinoma patients with hysterectomy and pelvic-paraaortic lymphadenectomy was not different from endometrioid endometrial cancer patients, according to the SEER analysis. Despite a low-grade disease, mucinous endometrial cancers could have lymphatic metastasis. On the other hand, lymphadenectomy is controversial for early-stage and low-grade cancers (3, 14). In this study, lymph node metastasis and LVSI were more common in the advanced stage group. However, we performed retroperitoneal, systematic lymphadenectomy to all patients as standard surgical therapy. There is a debate on the best surgical practice for early-stage mucinous endometrial carcinoma patients. Owusu-Darko *et al*. (15) performed a case-control study and did not find any difference between mucinous endometrial carcinoma and endometrioid endometrial carcinoma on overall survival and disease-free survival when treated with hysterectomy and bilateral salpingo-oophorectomy. Gungorduk *et al*. (16) also did not find any difference regarding the survival analysis of mucinous and endometrioid endometrial cancers. However, they suggested routine retroperitoneal lymphadenectomy for mucinous endometrial carcinoma patients because of the increased incidence of lymph node metastasis. Despite the tendency towards lymphatic dissemination, the risk of myometrial invasion does not increase significantly in mucinous endometrial carcinomas (7). It is a less aggressive form of endometrial cancer with similar management options as endometrioid endometrial carcinoma (3). Whorley *et al*. (17) showed an increased incidence of myometrial invasion for patients with mucinous endometrial carcinoma. Nevertheless the incidence of deep myometrial invasion was not higher, a finding also reported by Musa *et al*. (5). They did not find any significant difference for LVSI. In contrast to that study, LVSI was more common in the advanced stage group of our patient cohort.

Tumour markers are commonly used parameters of gynaecologic oncology practice. They play a role in the detection and monitoring of malignancies, especially ovarian cancer. CA-19.9 is a monosialoganglioside, associated with mucins in gastrointestinal adenocarcinomas (18) and CA-125 is known to be expressed in the coelomic epithelium, including the Mullerian epithelium, peritoneum, pleura, and pericardium (19). The functional rationale for the use of these markers has been proposed in various studies previously. There are many ongoing studies related to endometrial cancer and tumour markers. We found CA-125 and CA-19.9 significantly elevated in the group of advanced disease patients. However, the only significant parameter for patients with lymph node metastasis was CA-19.9. Neunteufel *et al*. (20) reported an association between well-differentiated endometrial adenocarcinomas and CA-19.9 positivity. Duk *et al*. (21) proposed a significance between stage and CA-125 for endometrial cancers. Kanat-Pektas *et al*. (22) defined elevated levels of CA-125 and CA-19.9 for endometrial carcinoma that was also correlated with tumour stage. However, non-selective elevations of these markers with various benign and malignant situations show the poorness of these markers. Baser *et al*. (23) found CA-125, CA-19.9, and CA-15.3 were significant in predicting postoperative adjuvant therapy need. CA-125 was especially important during this

management. Since the progressive increase of CA-125 from well to poorly differentiated carcinomas, Cherchi *et al*. (24) defined CA-125 as the most valuable marker for endometrial carcinomas.

The data regarding mucinous endometrial carcinomas are limited. The need of the tumour markers for the prediction of prognosis and prognosticators is controversial, and there is also controversy regarding sensitivity and specificity. On the other hand, CA-125 is a well-known marker with extensive literature data. In this article, CA-19.9 is proposed as a marker for mucinous endometrial carcinomas, and we have not found similar research.

CONCLUSION

In conclusion, mucinous endometrial carcinomas are generally low-grade and low-stage cancers with an increased risk of nodal metastasis. Pre-operative workup of CA-19.9 could predict both advanced disease and lymph node metastasis for these tumours.

REFERENCES

1. Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. J Clin Oncol 2006; **24:** 4783–91. PubMed PMID: 17028294.
2. Galic V, Schiavone MB, Herzog TJ, Holcomb K, Lewin SN, Lu YS, et al. Prognostic significance of mucinous differentiation of endometrioid adenocarcinoma of the endometrium. Cancer Investig 2013; **31:** 500–4. PubMed PMID: 23915075. PubMed Central PMCID: 4230693.
3. Rauh-Hain JA, Vargas RJ, Clemmer J, Clark RM, Bradford LS, Growdon WB, et al. Mucinous adenocarcinoma of the endometrium compared with endometrioid endometrial cancer: a SEER analysis. Am J Clin Oncol. 2014 Jan 2. PubMed PMID: 24390270.
4. Briet JM, Hollema H, Reesink N, Aalders JG, Mourits MJ, ten Hoor KA, et al. Lymphvascular space involvement: an independent prognostic factor in endometrial cancer. Gynaecol Oncol 2005; **96:** 799–804. PubMed PMID: 15721428.
5. Musa F, Huang M, Adams B, Pirog E, Holcomb K. Mucinous histology is a risk factor for nodal metastases in endometrial cancer. Gynaecol Oncol 2012; **125:** 541–5. PubMed PMID: 22410328.
6. Van Gorp T, Cadron I, Vergote I. The utility of proteomics in gynaecologic cancers. Curr Opin Obst Gynaecol 2011; **23:** 3–7. PubMed PMID: 21235022.
7. Ross JC, Eifel PJ, Cox RS, Kempson RL, Hendrickson MR. Primary mucinous adenocarcinoma of the endometrium. A clinicopathologic and histochemical study. Am J Surg Pathol 1983; **7:** 715–29. PubMed PMID: 6318581.
8. Blaustein A KR. Blaustein's pathology of the female genital tract. 5 ed. New York: Springer-Verlag; 2002.
9. Fujiwara M, Longacre TA. Low-grade mucinous adenocarcinoma of the uterine corpus: a rare and deceptively bland form of endometrial carcinoma. Am J Surg Pathol 2011; **35:** 537–44. PubMed PMID: 21378544.
10. Yoo SH, Park BH, Choi J, Yoo J, Lee SW, Kim YM, et al. Papillary mucinous metaplasia of the endometrium as a possible precursor of endometrial mucinous adenocarcinoma. Mod Pathol 2012; **25:** 1496–507. PubMed PMID: 22766790.
11. Nucci MR, Prasad CJ, Crum CP, Mutter GL. Mucinous endometrial epithelial proliferations: a morphologic spectrum of changes with diverse clinical significance. Mod Pathol 1999; **12:** 1137–42. PubMed PMID: 10619266.
12. Jalloul RJ, Elshaikh MA, Ali-Fehmi R, Haley MM, Yoon J, Mahan M, et al. Mucinous adenocarcinoma of the endometrium: case series and review of the literature. Int J Gynaecol Cancer 2012; **22:** 812–8. PubMed PMID: 22569105.
13. Melhem MF, Tobon H. Mucinous adenocarcinoma of the endometrium: a clinico-pathological review of 18 cases. Int J Gynaecol Pathol 1987; **6:** 347–55. PubMed PMID: 2826354.
14. Chan JK, Cheung MK, Huh WK, Osann K, Husain A, Teng NN, et al. Therapeutic role of lymph node resection in endometrioid corpus cancer: a study of 12,333 patients. Cancer 2006; **107:** 1823–30. PubMed PMID: 16977653.
15. Owusu-Darko S, Rauh-Hain JA, Horowitz NS, Goodman A, Schorge JO, Del Carmen MG. Comparison of outcomes in patients with early-stage mucinous endometrial cancer and those with endometrioid endometrial cancer, with and without adjuvant therapy. J Reprod Med 2014; **59:** 527–33. PubMed PMID: 25552123.
16. Gungorduk K, Ozdemir A, Ertas IE, Selcuk I, Solmaz U, Ozgu E, et al. Is mucinous adenocarcinoma of the endometrium a risk factor for lymph node involvement? A multicenter case-control study. Int J Clin Oncol 2014. PubMed PMID: 25380693.
17. Worley MJ, Jr., Davis M, Berhie SH, Muto MG, Feltmate CM, Berkowitz RS, et al. Mucinous differentiation does not impact stage or risk of recurrence among patients with grade 1, endometrioid type, endometrial carcinoma. Gynaecol Oncol 2014; **135:** 54–7. PubMed PMID: 25088333.
18. Scharl A, Crombach G, Vierbuchen M, Gohring U, Gottert T, Holt JA. Antigen CA 19–9: presence in mucosa of nondiseased mullerian duct derivatives and marker for differentiation in their carcinomas. Obstet Gynaecol 1991; **77:** 580–5. PubMed PMID: 2002982.
19. Bischof P. What do we know about the origin of CA 125? Eur J Obstet Gynaecol Reprod Biol 1993; **49:** 93–8. PubMed PMID: 8365529.
20. Neunteufel W, Bieglmayer C, Breitenecker G. CA19-9, CA125 and CEA in endometrial carcinoma tissue and its relation to hormone receptor content and histological grading. Arch Gynaecol Obstet 1988; **244:** 47–52. PubMed PMID: 2853610.
21. Duk JM, Aalders JG, Fleuren GJ, de Bruijn HW. CA 125: a useful marker in endometrial carcinoma. Am J Obstet Gynaecol 1986 Nov; **155:** 1097–102. PubMed PMID: 3465243.
22. Kanat-Pektas M, Yenicesu O, Gungor T, Bilge U. Predictive power of sexual hormones and tumour markers in endometrial cancer. Arch Gynaecol Obstet 2010; **281:** 709–15. PubMed PMID: 19777250.
23. Baser E, Gungor T, Togrul C, Turkoglu O, Celen S. Preoperative prediction of poor prognostic parameters and adjuvant treatment in women with pure endometrioid type endometrial cancer: what is the significance of tumour markers? Eur J Gynaecol Oncol 2014; **35:** 513–8. PubMed PMID: 25507418.
24. Cherchi PL, Dessole S, Ruiu GA, Ambrosini G, Farina M, Capobianco G, et al. The value of serum CA 125 and association CA 125/CA 19-9 in endometrial carcinoma. Eur J Gynaecol Oncol 1999; **20:** 315–7. PubMed PMID: 10475131.

ORIGINAL ARTICLE

Serum Red Blood Cell Distribution Width Level of Chronic Obstructive Pulmonary Disease Patients and the Characteristics of the Exacerbation

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ABSTRACT

Objective: *Red blood cell distribution width (RDW) is a measure of red blood cell size heterogeneity. The aim of this study is to evaluate the relationship between the RDW level and the characteristics of the chronic obstructive pulmonary disease (COPD) exacerbations.*

Methods: *The records of the hospitalised COPD patients were evaluated retrospectively. The demographic characteristics, duration of follow-up and the numbers of exacerbations and hospitalisations, duration of the hospital stay, need of non-invasive mechanic ventilation (NIMV) or intensive care unit (ICU) and laboratory findings were recorded.*

Results: *There were 35 women (18.5%), 154 men (81.5%), Totaling 189 patients whose mean age was 67.4 ± 10.6 years. The mean durations of follow-up and hospitalisation were, respectively, 2.85 ± 2.5 years and 8.5 ± 5.4 days. Forty-one of the patients (22%) were current smokers and 115 of them (61.8%) were ex-smokers. Nearly one-third of the patients (34.9%) had increased RDW levels. When the patients with an increased RDW level compared to normal ones, no statistically significant difference was found with respect to the gender, smoking history, smoking pack-years, numbers of the exacerbation and hospitalisation, duration of hospital stay and need of NIMV or ICU. However, it was found that the presence of exitus was significantly higher in patients with an increased RDW level (*p = *0.02).*

Conclusion: *This study suggested that an increased level of RDW might be related with increased mortality in COPD patients. Further long-term prospective studies with healthy controls and COPD patients in both stable and exacerbation periods are needed in order to evaluate clinical significance of these findings.*

Keywords: COPD, RDW, exacerbation, hospitalisation, mortality

INTRODUCTION

Red blood cell distribution width (RDW) is a measure of red blood cell size heterogeneity and it reflects the erythrocyte morphology (1). It is a component of complete blood count and it is used in the differential diagnosis of anaemia (2). Furthermore, systemic inflammation, ineffective erythropoiesis, nutritional deficiencies, bone marrow dysfunction or increased destruction can also cause a higher RDW. Recent evidence reports that acute or chronic disorders such as cardiovascular diseases (CVD), cancer, sepsis, chronic obstructive pulmonary disease (COPD), pulmonary thromboembolism (PTE), diabetes mellitus, liver and kidney failure are related with increased RDW levels (1–4).

It is also reported that higher RDW levels may reflect underlying chronic inflammation, which can result in an increased mortality (3). There is an association between increased RDW levels and high mortality risk in both general population and some diseases including CVD and COPD (2, 3). COPD is a systemic disease and inflammation has an important role on development of COPD. COPD-related inflammation may also impair erythropoiesis and cause increased RDW levels, as do other chronic inflammatory processes. However,

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there are limited studies evaluating the relation among COPD, mortality and RDW levels (1, 3).

Chronic obstructive pulmonary disease is a treatable and preventable systemic disease, characterised by persistent airflow limitation that is usually progressive and associated with an increased inflammatory response to noxious particles and gases (5). It is the fifth leading cause of death and estimated to be the third in 2030 worldwide (6). Exacerbations are an important cause of mortality and morbidity in COPD patients (7, 8). The aim of this study is to evaluate the relationship between the RDW level and the characteristics of the COPD exacerbations.

SUBJECTS AND METHODS

The medical records of the hospitalised patients with COPD exacerbation in the Pulmonary Diseases Clinic were evaluated retrospectively. The exacerbation of the COPD was defined as an acute event characterised by a worsening of the respiratory symptoms that was beyond normal day-to-day variations and led to a change in medication (5).

The demographic characteristics, smoking history, duration of follow-up and the numbers of exacerbations and hospitalisations, duration of the hospital stay, need of non-invasive mechanic ventilation (NIMV) or intensive care unit (ICU) were recorded. Laboratory findings including haemogram and artery blood gas analysis that were taken on the first day of hospitalisation were also recorded.

Exclusion criteria

Patients with other lung diseases such as tuberculosis, bronchiectasis and interstitial lung disease were excluded. Patients with iron or vitamin deficiencies (such as B12 or folate) and recent transfusions were also excluded from the study.

Laboratory analysis

The red cell distribution width was assessed by flow cytometric analysers as the part of the routine full blood count. The red blood cell distribution width was calculated as (standard deviation of red cell volume/mean cell volume) × 100. The normal range was between 12.2% and 17.2% in our laboratory.

Statistical analysis

Statistical analyses of the study were performed using IBM SPSS for Windows version 20.0 (SPSS, Chicago, IL, USA). Categorical variables were expressed as counts (percentage). Numeric variables were expressed as mean and standard deviation. Comparisons of categorical variables between the groups were performed using the Chi-square test. A two-sided p-value < 0.05 was considered statistically significant.

RESULTS

There were 35 women (18.5%), 154 men (81.5%), totalling 189 patients. The mean age was 67.4 ± 10.6 years. The mean durations of follow-up and hospitalisation were, respectively, 2.85 ± 2.5 years and 8.5 ± 5.4 days. Forty-one of the patients (22%) were current smokers and 115 of them (61.8%) were ex-smokers. Respiratory infections (72%) were the most common causes of the exacerbations. Demographic data, the characteristics of exacerbation and laboratory findings of the patients are shown in Tables 1 and 2.

It was found that the level of RDW was increased in one-third of the patients (34.9%). Retrospective analyses of 189 COPD patients revealed that the RDW level had a mean value of 16.95 ± 2.2. The comparison of exacerbation characteristics and laboratory parameters according to the RDW levels are shown in Table 3.

When the patients with increased RDW level were compared to normal ones, no statistically significant difference was found with respect to the gender, smoking history, smoking pack-years, numbers of the exacerbation and hospitalisation, duration of hospital stay and need of NIMV or ICU (Table 4). However, it was found that the presence of exitus was significantly higher in patients with increased RDW level ($p = 0.02$) (Figure).

DISCUSSION

This study demonstrated that nearly one-third of the hospitalised COPD patients with the exacerbation had increased RDW levels. There were no statistically significant differences of gender, smoking history, smoking pack-years, numbers of the exacerbation and hospitalisation, duration of hospital stay and need of NIMV or ICU in patients with increased RDW level compared to normal ones. However, it was found that the presence of exitus was significantly higher in patients with increased RDW level.

The red blood cell distribution width (RDW) is a simple and cheap parameter, which is a component of the haemogram. It reflects the degree of heterogeneity of erythrocyte volume. It is also known as anisocytosis. The red blood cell distribution width is used for differential diagnosis of anaemias (2). Beside this, RDW is a recently recognised biomarker of adverse outcome in

Table 1: Demographic characteristics of the patients

		n	%
Gender	Female	35	18.5
	Male	154	81.5
Smoking history	Non-smoker	30	16.1
	Current smoker	41	22
	Ex-smoker	115	61.8
RDW	Normal RDW	123	65.1
	Increased RDW	66	34.9
NIMV	(–)	114	60.3
	(+)	75	39.7
ICU	(–)	175	92.6
	(+)	14	7.4
Exitus	(–)	177	93.7
	(+)	12	6.3

ICU = intensive care unit; NIMV = non-invasive mechanic ventilation; RDW = red blood cell distribution width.

Table 2: The characteristics of exacerbation and laboratory parameters

	Min	Max	Mean ± SD
Age, years	35	93	67.4 ± 10.6
Smoking pack/year	0	140	42.8 ± 29.9
The number of exacerbation, n	1	20	2.9 ± 3.4
The number of hospitalisation, n	1	12	2.1 ± 2.1
Duration of hospitalisation, day	1	34	8.5 ± 5.4
Haemoglobin g/dL	8.9	18.1	13.03 ± 1.7
Haematocrit, %	25.2	55.1	39.8 ± 5.3
RDW	13.4	27.1	16.95 ± 2.2
CRP, mg/dL	0.04	47.82	8.04 ± 10.1
$PaCO_2$, mmHg	22	92	48.7 ± 14.2
SO_2, %	65.6	99.1	90.1 ± 10.1

CRP = C-reactive protein; RDW = red blood cell distribution width; SD = standard deviation.

Table 3: The comparison of exacerbation characteristics and laboratory parameters according to RDW levels

	Normal RDW	Increased RDW	*p*-value
Age, years	66.8 ± 10.8	68.6 ± 9.97	0.15
Smoking pack/year	40.8 ± 28.6	46.3 ± 32	0.7
The number of exacerbation, n	2.9 ± 3.4	3.02 ± 3.4	0.1
The number of hospitalisation, n	2.05 ± 1.9	2.2 ± 2.3	0.7
Duration of hospitalisation, day	8.61 ± 5.8	8.4 ± 4.5	0.8
CRP, mg/L	8.6 ± 10.7	7.02 ± 8.9	0.42
PaO_2, mmHg	67.7 ± 18.9	66.3 ± 16.7	0.08
$PaCO_2$, mmHg	47.2 ± 13.6	51.03 ± 15.1	0.007
SO_2, %	90.7 ± 7.8	89.3 ± 7.6	0.47

CRP = C-reactive protein; RDW = red blood cell distribution width.

various acute and chronic conditions (9). An increased RDW is related with both impaired erythropoiesis and abnormal red blood cell survival, which may be attributed to a variety of underlying metabolic abnormalities

Table 4: Demographic characteristics of the patients according to RDW level

		Normal RDW		Increased RDW		*p*-value
		n	%	n	%	
Gender	Female	19	15.4	16	24.2	0.13
	Male	104	84.6	50	75.8	
Smoking history	Non-smoker	19	15.8	11	16.7	0.8
	Current smoker	25	20.8	16	24.2	
	Ex-smoker	76	63.3	39	59.1	
NIMV	(–)	80	65	34	51.5	0.07
	(+)	43	35	32	48.5	
ICU	(–)	116	94.3	59	89.4	0.07
	(+)	7	5.7	7	10.6	
Exitus	(–)	119	96.7	58	87.9	0.02
	(+)	4	3.3	8	12.1	

ICU = intensive care unit; NIMV = non-invasive mechanic ventilation; RDW = red blood cell distribution width.

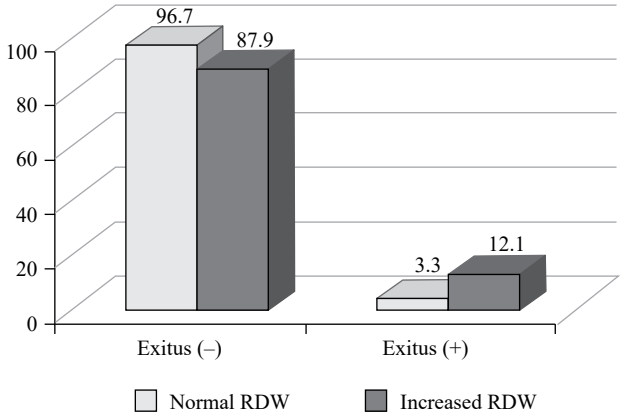

Figure: Comparison of RDW levels according to presence of exitus. RDW = red blood cell distribution width.

such as oxidative stress, inflammation, erythrocyte fragmentation and alteration of erythropoietin function (2).

Anisocytosis can also see in some disorders such as cardiovascular disease, cancer, sepsis, diabetes, liver/kidney failure and respiratory diseases including community acquired pneumonia (CAP), venous thromboembolism and COPD (2, 4, 10–12). The studies evaluating the relation between COPD and RDW levels are limited (1, 3). In a retrospective analysis of stable COPD patients, it was revealed that RDW is positively correlated with C-reactive protein (CRP), right ventricular dysfunction and pulmonary arterial hypertension (3). In a multivariable logistic regression analysis, the presence of high RDW was found to be only for independent parameter predicting right ventricle (RV) failure in COPD patients and it is suggested that RDW may be used to identify COPD patients with RV failure (13).

Chronic obstructive pulmonary disease is a treatable and preventable systemic disease characterised by persistent airflow limitation that is usually progressive (5). It is one of the most important causes of mortality and morbidity worldwide. Particularly in older-age groups in the United States, it is among the 10 most important disease-causing hospitalisations and death with increasing prevalence day-by-day (14). Exacerbations are the most frequent cause of morbidity, hospital admission and mortality in COPD (15). The exacerbation of the COPD is defined as an acute event characterised by worsening of the respiratory symptoms that was beyond normal day-to-day variations and led to a change in medication (5). Respiratory failure can be seen in the exacerbation of COPD. The arterial blood gas analysis is an important tool for defining respiratory failure and to start NIMV therapy in COPD patients. The relation between RDW and arterial blood gas analysis parameters of COPD patients was also investigated. It is reported that oxygen saturation is significantly lower for patients with an elevated RDW (1). In our study, it was also found that mean PaO_2 and oxygen saturation values were lower in patients with increased RDW levels compared to normal ones. However, the difference was not statistically significant. Beside this, mean $PaCO_2$ was significantly higher in patients with increased RDW level compared to normal ones (51.03 ± 15.1 *vs* 47.2 ± 13.6, $p = 0.007$). It suggests that RDW level may be useful for predicting hypercapnic COPD patients who needs NIMV therapy.

The stage of COPD is assessed by the pulmonary function test, health status and presence of exacerbation/hospitalisation (5). It reflects the severity of disease. There is a study evaluating the relation between RDW level and the stage of disease. The highest RDW is observed in the very severe stage ($p < 0.001$) of COPD patients in the study by Tertemiz *et al.* (1). In our study, it was not possible to compare the RDW levels according to stages of disease due to its retrospective design. There was a lack of data about health status assessed by modified Medical Research Council (mMRC) Questionnaire or COPD assessment test (CAT) due to the retrospective nature of this study. Furthermore, most of the patients were not able to perform the pulmonary function test since they had a severe dyspnoea due to exacerbation.

The association between RDW level and length of stay (LOS) in hospital is also investigated in recent studies (11, 16). The red blood cell distribution width (RDW) is associated with length of stay in hospital and use of vasopressors in hospitalised patients with CAP (11). Greater initial RDW and change of RDW during the heart failure hospitalisation are associated with longer LOS, suggesting that both initial RDW and change of RDW during the hospitalisation may be helpful in personalising prognosis and treatment (16). In contrast, there was no significant difference of LOS in the hospital between the groups in our study. It is thought that study populations including different diseases might be responsible for opposite results.

The baseline RDW and an increase in RDW during the hospitalisation are also compared in patients with sepsis and heart failure (16, 17). It is reported that there is an increase in RDW from baseline during the first 72 hours after the hospitalisation, and it is significantly associated with adverse clinical outcomes in sepsis. Therefore, it is suggested that a combination of baseline RDW value and an increase in RDW can be a promising independent prognostic marker in patients with severe sepsis or septic shock (17). It is also suggested that both initial RDW and change of RDW during the hospitalisation may be helpful in personalising prognosis and treatment of patients with heart failure (16). However, it was not possible to evaluate the role of RDW changes during the hospitalisation on adverse clinical outcomes due to retrospective design of this study.

A potential independent association is recently demonstrated between high RDW levels and the risk of all-cause mortality in critically ill patients, although the underlying mechanism is still unclear (17). An elevated RDW has been associated with adverse outcomes of heart failure, infective endocarditis, VTE and idiopathic pulmonary hypertension (9,18). The RDW is recognised as a mortality marker in patients with CVD (3). It is also reported that baseline RDW is significantly associated with incidence of fatal cardiac events (19). An increased RDW conveys an important information for both short- and long-term prognosis (3). Greater initial RDW and change of RDW during the heart failure hospitalisation are associated with 30-day mortality (16). The RDW is found to be associated with 30-day mortality in hospitalised patients with CAP (11). Even more importantly, the value of RDW is now being regarded as a strong and independent risk factor for death in the general population (2). Furthermore, the studies evaluating the role of RDW on mortality in COPD patients are also support these findings (1, 3). Elevated RDW levels are associated with an increased mortality risk in stable COPD patients (3). It is suggested that there is a correlation between survival of COPD patients and RDW levels (1). Also, the presence of exitus was significantly higher in patients with an increased RDW level in our study as similar to the literature.

There were several limitations of this study that should be considered. Firstly, it was a retrospective investigation, and data collection was based on medical records. Secondly, it reflected the RDW levels of only hospitalised patients with COPD exacerbation. So, the study group did not include outpatients and study population was relatively small. Furthermore, it was not possible to compare the RDW results of the patients in stable or exacerbation periods. Thirdly, only baseline RDW levels of the patient were recorded, so comparing the role of changes of RDW levels during the hospitalisation on adverse clinical outcomes was not possible. Fourthly, it was not possible to assess the patient's health status by modified Medical Research Council (mMRC) Questionnaire or COPD assessment test (CAT) and to evaluate the new classification of COPD because of its retrospective nature. So, the severity of the disease was not classified.

CONCLUSION

Early detection and effective treatment of exacerbations are key factors that may reduce in hospital admissions. The RDW is a simple, easy to perform and cheap parameter. It might be useful for predicting hypercapnic COPD patients who needs NIMV therapy in the exacerbation period. Also, it is suggested that an increased level of RDW might be related with increased mortality in COPD patients. Further long-term prospective studies with healthy controls and COPD patients in both stable and exacerbation periods are needed in order to evaluate clinical significance of these findings.

AUTHORS' NOTE

SAB conceived paper, oversaw data collection, conducted data analysis, wrote manuscript and approved final version. T Onyilmaz participated in study design, data collection, wrote manuscript and approved final version. EKU participated in study design, data collection, and revision of manuscript and approved final version. I Basyigit participated in study design, interpretation of data and revision of manuscript and approved final version. HB and FY participated in study design and interpretation of data, critically revised manuscript and approved final version. The authors declare that they have no conflicts of interest.

REFERENCES

1. Tertemiz KC, Ozgen Alpaydin A, Sevinc C, Ellidokuz H, Acara AC, Cimrin A. Could "red cell distribution width" predict COPD severity? Rev Port Pneumol 2016; **22:** 196–201.
2. Salvagno GL, Sanchis-Gomar F, Picanza A, Lippi G. Red blood cell distribution width: a simple parameter with multiple clinical applications. Crit Rev Clin Lab Sci 2015; **52:** 86–105.
3. Seyhan EC, Özgül MA, Tutar N, Ömür I, Uysal A, Altin S. Red blood cell distribution and survival in patients with chronic obstructive pulmonary disease. COPD 2013; **10:** 416–24.
4. Zöller B, Melander O, Svensson P, Engström G. Red cell distribution width and risk for venous thromboembolism: a population-based cohort study. Thromb Res 2014; **133:** 334–9.
5. Global Initiative for Chronic Obstructive Lung Disease (GOLD). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: updated 2014. Available from: http://www.goldcopd.org/uploads/users/files/GOLD_Report2014 Feb07
6. Celli BR, Mac Nee W, ATS/ERS Task Force. Standards for the diagnosis and care of patients with COPD: a summary of the ATS/ERS position paper. Eur Respir J 2004; 23: 932–46. http://www.who.int/respiratory/copd/burden/en/ [accessed 06.07.14].
7. Miravitlles M, Izquierdo I, Herrejón A, Torres JV, Baró E, Borja J et al. COPD severity score as a predictor of failure in exacerbations of COPD. The ESFERA study. Respir Med 2011; **105:** 740–7.
8. Guray Y, Ipek EG, Guray U, Demirkan B, Kafes H, Asarcikli LD et al. Red cell distribution width predicts mortality in infective endocarditis. Arch Cardiovasc Dis 2014; **107:** 299–307.
9. Abul Y, Ozsu S, Korkmaz A, Bulbul Y, Orem A, Ozlu T. Red cell distribution width: a new predictor for chronic thromboembolic pulmonary hypertension after pulmonary embolism. Chron Respir Dis 2014; **11:** 73–81.
10. Lee JH, Chung HJ, Kim K, Jo YH, Rhee JE, Kim YJ et al. Red cell distribution width as a prognostic marker in patients with community-acquired pneumonia. Am J Emerg Med 2013; **31:** 72–9.
11. Koma Y, Onishi A, Matsuoka H, Oda N, Yokota N, Matsumoto Y et al. Increased red blood cell distribution width associates with cancer stage and prognosis in patients with lung cancer. PLoS One 2013; **8:** e80240.
12. Sincer I, Zorlu A, Yilmaz MB, Dogan OT, Ege MR, Amioglu G et al. Relationship between red cell distribution width and right ventricular dysfunction in patients with chronic obstructive pulmonary disease. Heart Lung 2012; **41:** 238–43.
13. Mannino DM. COPD: epidemiology, prevalence, morbidity and mortality, and disease heterogeneity. Chest 2002; **121:** 121S–6S.
14. Miravitlles M, Guerrero T, Mayordomo C, Sánchez-Agudo L, Nicolau F, Segú JL. Factors associated with increased risk of exacerbation and hospital admission in a cohort of ambulatory COPD patients: a multiple logistic regression analysis. Respiration 2000; **67:** 495–501.
15. Muhlestein JB, Lappe DL, Anderson JL, Muhlestein JB, Budge D, May HT et al. Both initial red cell distribution width (RDW) and change in RDW during heart failure hospitalization are associated with length of hospital stay and 30-day outcomes. Int J Lab Hematol 2016; **38:** 328–37.
16. Kim CH, Park JT, Kim EJ, Han JH, Han JS, Choi JY et al. An increase in red blood cell distribution width from baseline predicts mortality in patients with severe sepsis or septic shock. Crit Care 2013; **17:** R282.
17. Ellingsen TS, Lappegård J, Skjelbakken T, Brækkan SK, Hansen JB. Red cell distribution width is associated with incident venous thromboembolism (VTE) and case-fatality after VTE in a general population. Thromb Haemost 2015; **113:** 193–200.
18. Borné Y, Smith JG, Melander O, Engström G. Red cell distribution width in relation to incidence of coronary events and case fatality rates: a population-based cohort study. Heart 2014; **100:** 1119–24.

ORIGINAL ARTICLE

Thyroid Dysfunction Related to Interferon in Turkish Patients with Viral Hepatitis: Incidence and Influencing Factors

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ABSTRACT

Objective: *Interferon therapy is widely used for patients with chronic viral hepatitis B and C (CHB-C). It is known that thyroid gland dysfunction (TD) may develop secondary to interferon treatment, but different frequencies of TD development are reported in the literature. For this reason, we aimed to investigate the incidence of TD developing secondary to interferon treatment in patients with CHB-C, treated in our clinic, and identify possible influencing factors.*

Methods: *A total of 93 CHB-C patients treated with interferon were included in this study. Tests of thyroid functions (thyroid-stimulating hormone (TSH), FT3, FT4), thyroid auto-antibodies (Anti-Tg, Anti-TPO) were done before starting interferon treatment, during, and after treatment. Patients who did not have normal thyroid gland functions were not included in this study.*

Results: *TD was observed to develop in a total of 20 patients (21.5%). TD had developed in the first six months after initiation of interferon therapy. In 18.3% of these patients, the condition was temporary, while 3.2% of patients required treatment for TD. The most frequently seen condition was the subclinical types. Age (over 40 years) (OR: 7.25 95% CI: 1.46, 35.80,* p *= 0.015) and sex (female) (OR: 5.83 95% CI: 1.31, 25.76,* p *= 0.020) were found to be statistically significant independent risk factors in TD development.*

Conclusion: *Although TD secondary to interferon treatment in patients with CHB-C may develop in a substantial proportion of cases, most of these cases are temporary and do not require TD treatment. Independent risk factors in TD development were found to be sex and age.*

Keywords: Chronic viral hepatitis, interferon, thyroid gland dysfunction

INTRODUCTION

Chronic viral hepatitis consists of a group of diseases widely seen all over the world, and which may result in hepatic failure, cirrhosis, or hepatocellular carcinoma, while chronic viral hepatitis B and C (CHB-C) are the most frequently seen types.

Although there are newly developed anti-viral agents with direct effects in CHB-C treatment, interferon (α-2a,-2b) and antiviral nucleoside analogues (ribavirin) are routinely accepted as standard therapeutic agents.

Among these standard agents, some unwanted effects, such as haematologic changes (anaemia, neutropenia, thrombocytopenia), flu-like symptoms, fever, depression and endocrinologic disorders may be seen due to interferon (1–3). The most frequently seen and known effect on the endocrinologic system, apart from the liver, is dysfunction of the thyroid gland. In many studies on this dysfunction, different results were reported on the incidence of thyroid gland dysfunction (TD) between 3 and 35% (4–7).

Although the TD is believed to be mediated by auto-antibodies, the exact mechanism is not known. In some studies, TD was shown to develop more frequently in patients with chronic hepatitis C (CHC), in comparison to patients with chronic hepatitis B (CHB), which suggested that causes of viral origin may be effective in the development of TD (8).

In various research studies, sex presence of thyroid auto-antibodies, and ethnic factors were shown to be

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associated with TD developing due to interferon (IFN) (7–11).

CHB-C is a frequently encountered health problem in our country and similar to global practice, interferon is widely used in its treatment. When the literature is examined, the number of studies showing the incidence of TD developing due to IFN use in patients with CHC in Turkey is scarce, and data evaluating CHB-C patients together is missing. For this reason, we aimed to investigate the incidence of TD developing secondary to interferon use in patients with CHB-C, and associated risk factors.

SUBJECTS AND METHODS

Patients

Patient file records of individuals diagnosed with CHB-C, and who were treated and followed-up between 2010 and 2015 in a private SANKO hospital, were examined retrospectively. The main inclusion criteria of this study were: age over 18 years, being treated with IFN due to CHB-C for at least four weeks, absence of known thyroid disease before IFN treatment, and thyroid gland functions in the normal range. The patients with HIV infection, presence of other hepatic disorders (Wilson's disease, alcoholic-autoimmune liver disease), and those with abnormal thyroid gland function tests before treatment were excluded from the study.

The diagnosis of CHB-C was based on clinical, laboratory, USG and histopathological findings. Hepatitis B and C markers (HBsAg, HBeAg, anti-HBcIgM, anti-HBcIgG, anti-HBe, anti-HBs, anti-hepatitis C [HCV]) were measured from patient peripheral blood samples by microparticle ELISA method (Abbott-Architec i2000sr, Malvern, Pennsylvania, USA), measurement of levels of HBV-DNA, HCV-RNA and HCV genotyping were done with real-time polymerase chain reaction device.

The patients who were included in the study were classified into two groups according to viral types as follows:

Group 1

A total of 46 patients with a diagnosis of CHB were designated as group 1, 22 were females and 24 were males, with an age range of 20–61 years (mean 37.4 ± 10.3 years). In patients with CHB, pegylated interferon alpha-2b were administered *via* the subcutaneous route (<40 kg: 50 mcg/week, 40–64 kg: 80 mcg/week, 65–75 kg 100 mcg/week, 76–85 120 mcg/week, >80 kg 150 mcg/week) for a period of 48 weeks.

Group 2

A total of 47 patients with a diagnosis of CHC were designated as group 2. Of these, 16 patients were females and 31 were males, with an age range of 18–71 years (mean 52.6 ± 12.5 years), 44 patients (93.6%) were of the genotype1b, while the other 3 patients had genotypes 2, 3 and 4 subtypes.

In patients with CHC, pegylated interferon alpha-2b was administered *via* the subcutaneous route (< 40 kg: 50 mcg/week, 40–64 kg: 80 mcg/week, 65–75 kg 100 mcg/week, 76–85 120 mcg/week, > 80 kg 150 mcg/week) and ribavirin was given via the oral route (< 64 kg 800 mg/day, 65–85 kg 1000 mg/day, > 85 kg 1200 mg/day) as a combination therapy. This combination treatment was given for 48 weeks for genotypes 1 and 4, while it was given for 24 weeks for genotypes 2 and 3.

Evaluation of thyroid functions

Medical history query, physical examination, and tests of thyroid functions (free triiodothyronine (FT3), free thyroxin (FT4), thyroid-stimulating hormone (TSH)) and thyroid auto-antibodies (anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG)) were done for all patients before IFN treatment.

FT3, FT4, and TSH measurements were done by ultra-sensitive immune chemoluminescence non-competitive assay (ICMA) (Immmulite 2500 DPC, Siemens, Erlangen, Germany).

The reference range was 0.55–4.78 mIU/ml for TSH; 0.8–2.0 ng/dL for FT3; 0.89–1.76 ng/dl for FT4; <35 Iu/ml for anti-TPO; and <40 Iu/ml for anti-TG.

During treatment with IFN, the histories of the patients were evaluated every 3 months, with physical examination and TSH measurements. Those with an abnormal TSH level were considered as patients in whom TD had developed and levels of FT3, FT4, anti-TPO, and anti-TG were measured. These patients were followed-up at more frequent (every 6weeks) intervals.

Patients in whom TD had developed were classified according to tests of thyroid functions as follows: overt hypothyroidism (TSH level over normal and FT3–FT4 levels under normal limits), subclinical hypothyroidism (TSH level over normal limits and FT3–FT4 in normal limits), subclinical hyperthyroidism (TSH level below normal limits and FT3–FT4 levels in the normal range) and overt hyperthyroidism (TSH level below the normal limits and FT3–FT4 level over the normal limits).

While treatment of patients in whom overt hypothyroidism or overt hyperthyroidism have developed was started immediately, appropriate treatment for patients

in whom subclinical hypothyroidism or subclinical hyperthyroidism had developed was decided according to symptoms, physical examination findings and differences in laboratory measurements.

Statistical analysis

Analysis of the data was done with PASW® Statistics 18 software (SPSS Inc., Chicago, IL, USA). In the evaluation of data, mean, standard deviation, median, minimal and maximal values were used as descriptive statistical methods. Shapiro–Wilk test was used to determine the normal distribution of the data. Inter-group comparisons for categorical data were done with chi-square test, and student *t*-test was used for continuous variables. Logistic regression analysis was done in order to define independent factors associated with TD development; $p < 0.05$ was considered as statistically significant.

RESULTS

A total of 93 patients with CHB-C (47 CHC, 46 CHB) who met the inclusion criteria were included in this study. Thirty-eight of them were females (F), fifty-five were males (M), aged between 18 and 71 years (mean; 44.8 ± 13.7 years).

Of these 93 patients, TD development was observed in 20 (15F, 5M; 21.5%). Eleven of them (7 F, 4 M) had CHB, and 9 (8 F, 1 M) had CHC, with an age range of 30–71 years (mean: 49.7 ± 11.5 years). While the frequency of TD occurrence did not show statistically significant differences in terms of type of chronic viral hepatitis ($p = 0.57$), it was significantly higher among females in group 2 and in the whole of the group (group 1: $p = 0.2$, group 2: $p = 0.0001$, whole study group; $p = 0.0004$).

The mean age of patients with CHB in group 1 was 37.4 ± 10.3 years, and the mean age of patients with CHC in group 2 was 52.6 ± 12.5 years, and this difference between the groups was statistically significant ($p < 0.00001$). In patients in whom TD had developed, statistically significant differences were not found in terms of age in their groups and in the whole study group ($p = 0.06, 0.09, 0.07$).

Thyroid gland dysfunction (TD) was detected in the 3rd month after initiation of therapy in 10 patients, in the 6th month in 4 patients, and in the 12th month in 6 patients. In other words, TD had developed in the first 6 months after initiation of interferon therapy in 14 patients (70%). Nine of them had CHB, and 5 had CHC, with no statistically significant differences between the two groups ($p = 0.2$).

At the time of first diagnosis, the type of TD was overt hyperthyroidism in 1 patient, subclinical hyperthyroidism in 9 patients, subclinical hypothyroidism in 7 patients, and overt hypothyroidism in 3 patients. Among the 14 patients who were diagnosed to have TD in the first 6 months, 7 had subclinical hyperthyroidism, 5 had subclinical hypothyroidism, 1 had overt hyperthyroidism, and 1 had overt hypothyroidism.

Patients in whom TD had developed were followed up for 24 months after IFN therapy was completed. At the end of the 24th month, 17 patients (13 F, 4 M) in whom TFTs had returned to normal were considered to have temporary type, and 3 patients (2 F, 1 M) in whom TFTs had not returned to normal were considered to have permanent type TD.

Among 17 patients with temporary type TD (after IFN therapy was completed) TFTs returned to normal in the 6th month in 9 patients, in the 12th month in 6 patients, and in the 18th month in 2 patients. Medication use was needed in only 3 patients (2 with overt hypothyroidism, 1 with overt hyperthyroidism) during the follow-up period.

Before IFN therapy, thyroid autoantibodies (anti-TPO and/or anti-TG) were elevated in 9 of 93 patients (7 with CHC and 2 with CHB). Among the 20 patients who developed TD, thyroid autoantibodies were initially elevated in 5 patients (3 with CHC and 2 with CHB), and this difference was statistically significant ($p = 0.008$). Of the 3 patients with permanent-type TD (2 F, 1 M), one patient with CHB had overt hypothyroidism, while the other two patients (male with CHB and female with CHC) had subclinical hyperthyroidism.

Genotyping of patients with CHC was done and 44 (93.6%) were found to have genotype 1b while 3 patients had a different genotype (genotypes 2, 3 and 4). All of the patients in whom TD had developed were in the 1b genotype.

In the 3-year follow-up of patients with CHB, in terms of hepatitis treatment, a complete response was observed in 26 patients (56.5%), 13 patients had a recurrence and 7 patients were unresponsive to treatment, while among 11 patients in whom TD had developed a complete response was observed in 5 patients (45%), recurrence in 5 patients and 1 patient was unresponsive to treatment. There were no statistically significant differences between patients in whom TD had or had not developed in terms of response to hepatitis treatment ($p = 0.33$).

In the 3-year follow-up of patients with CHC, in terms of hepatitis treatment, a complete response was

observed in 29 patients (61%), recurrence was observed in 10 patients and 8 patients were observed to be unresponsive to treatment, while among 9 patients in whom TD had developed, 5 (55%) showed a complete response, 3 showed recurrence and 1 patient was unresponsive to treatment. There were no statistically significant differences between patients in whom TD had or had not developed in terms of response to hepatitis treatment ($p = 0.58$).

Some of the data showing the findings that were mentioned are presented in Table 1.

Table 1: Demographic, clinical and laboratory characteristics of the patients and a comparison

Feature	TD (+) (N = 20)	TD (−) (N = 73)	*p* value	All (N = 93)
Age (mean-SD), years	49.7 ± 11.5	44.6 ± 13.5	> 0.05[a]	44.8 ± 13.7
Sex (male/female)	5/15	50/23	< 0.05[b]	55/38
Viral hepatitis type (CHB/CHC)	11/9	35/38	> 0.05[b]	46/47
Presence of thyroid auto-antibodies (yes/no)	5/15	4/69	< 0.05[b]	9/84
Response to IFN treatment (response/ recurrence/no response)	10/8/2	45/15/13	> 0.05[b]	55/23/15
CHB (genotype)	11 (–)	35 (–)	–	46 (–)
CHC (genotype 1b/ other genotypes)	9 (9/0)	38 (35/3)	–	47 (44/3)

N = patient number; TD: thyroid gland dysfunction; SD = Standard deviation; CHB = chronic hepatitis B; CHC = chronic hepatitis C.
[a] = According to the independent *t*-test.
[b] = According to the chi-square test.

All of the study group (CHB-C) and both groups (CHB and CHC) were separately examined with multiple regression analysis in terms of the association of independent factors, such as sex, age (below 40 years and above 40 years), thyroid auto-antibodies (present or absent), viral type, and response to hepatitis treatment with the development of TD. Among these, age and sex were statistically significant variables and age, over 40 years, (OR: 7.25 95% CI: 1.46, 35.80, $p = 0.015$) and female sex (OR: 5.83 95% CI: 1.31–25.76, $p = 0.020$) were found to be independent risk factors in TD development.

Evaluation of risk factors that could be associated with thyroid dysfunction with multiple regression analysis are presented in Table 2.

Table 2: Evaluation of risk factors that may be associated with thyroid dysfunction with multiple regression analysis

Variable	OR	95% CI	*p* value
Age[a]	7.25	1.46–35.80	0.015
Sex (female)	5.83	1.31–25.76	0.020
Viral type	0.0345	0.09–1.27	0.111
Thyroid auto-antibodies	2.24	0.41–12.01	0.345
Response to treatment[b]	1.41	0.36–5.45	0.614

OR = odds ratio.
[a]: < 40 and ≥ 40 years.
[b]: Yes or no.

DISCUSSION

When the literature is reviewed (4–7), incidence of TD developing secondary to IFN therapy in patients with chronic viral hepatitis is observed to be in a wide range, such as 3%–35%. These different results may be related to many factors, such as genetic predispositions of patients included in studies, regional-ethnic differences, different TD definitions, treatment regimes, viral types and genotypes. The incidence of TD in patients with CHB-C treated with PEG-IFN was 21.5% (23.9% for CHB and 19.5% for CHC) in the present study. Although this result is higher than that reported by a meta-analysis (13) (approximately 6%), it may be considered similar to the incidence (16.8%) found in the study by Barut *et al.* (12) in Turkey.

Although TD is believed to develop via auto-antibodies during IFN therapy, the exact mechanism is still unknown. In some studies, it was shown that TD more frequently developed in patients with CHC in comparison to patients with CHB, which has suggested that causes of viral origin may be effective in TD development (8). There are also studies which had suggested that CHC infection may be responsible for thyroid autoimmunity, independently from IFN therapy (14, 15). On the other hand, TD was observed to develop more frequently, albeit not statistically significant, in patients with CHB in our study (23.9% for CHB and 19.5% for CHC). This result may have originated from the absence of homogeneity between two patient groups in terms of independent risk factors, such as sex and age, which are associated with TD development (this is partly due to comparison of patients with CHB and CHC not being a primary aim of our study).

When patients in whom TD have developed are evaluated, TD is observed to develop in the first 3 months after initiation of IFN therapy, while this proportion is 70% in the 6th month. Although most of these are patients with CHB (9 with CHB and 5 with CHC), a significant difference was not found between these two groups. We found the rate of TD development in patients with CHC in the 6th month to be 55%. While this is a lower rate than that found by Yan *et al*. (7) in patients with CHC (88.2%), it

is seen that most of TD development during IFN therapy in our study, both in the whole group and in patients with CHC, starts in the first six months.

At first diagnosis, 45% of the patients had subclinical hyperthyroidism, and 35% had subclinical hypothyroidism, in terms of type of TD. In other words, 80% of patients had a subclinical form of TD. When subclinical and overt types of TD were counted under one heading as hypothyroidism and hyperthyroidism, they included equal numbers of patients. While hypothyroidism was the most frequently encountered form in many of the studies (7, 16, 17), hyperthyroidism was most frequently reported in the study by Hsieh *et al*. (18).

Patients in whom TD had developed were followed up for 24 months after IFN treatment was completed. At the end of the 24th month, TFTs had returned to normal in 17 patients (85%), while 3 patients (15%) still had TD. Of these 17 patients (after IFN therapy was completed), TFTs had returned to normal in 9th at the 6th month, in 6 patients at the 12th month, and in 2 patients at the 18th month. Use of medications was required in only 3 patients (2 with overt hypothyroidism and 1 with overt hyperthyroidism) during the follow-up. In patients in whom TD persisted at the end of 24 hours, one had overt hypothyroidism and the other two had subclinical hyperthyroidism, and their appropriate treatment and controls were continuing.

If we summarise these results, it may be told that TD is mostly in subclinical forms and improved in time without requiring treatment. These findings are similar to former studies (7, 19).

As we have mentioned before, sex and age are among the factors that are effective in TD development. Especially in terms of sex, the presence of a more reactive immune system in females, in comparison to males, may explain more frequent occurrence of TD associated with IFN, but while many studies support this (7, 10, 11, 18), some studies did not find a significant association (12, 20, 21). Age (over 40 years) and female sex were found to be statistically independent risk factors in TD development in our study.

When former studies are examined, results showing that factors such as CHC genotype, thyroid auto-antibodies, and response to IFN therapy may be effective in TD development are observed to be reported (12, 16–22).

Genotype evaluation of patients with CHC in our study was done; 93.6% of all patients and all of the patients in whom TD had developed were found to have genotype 1b. Genotype 1b is the most prevalent genotype in patients with CHC in our country, and our study group is in accordance with this. Pavan *et al*. (23) have reported that TD development was more frequent in CHC genotype 1. As most of our study group had genotype 1b, a statistical analysis could not be done between genotype and TD development.

The prevalence of thyroid auto-antibodies was reported to be between 20 and 30% in patients with CHC, and the presence of anti-TPO or anti-TG antibodies was reported to be associated with TD developing due to IFN (11, 24, 25). Thyroid auto-antibodies measured before IFN therapy was 9.6% in the whole group (14.9% in patients with CHC), and this ratio was 25% in patients in whom TD had developed. This difference was statistically significant, but the presence of thyroid auto-antibodies was not found to be significant as an independent risk factor in multiple regression analysis. Considering the studies reporting the prevalence of thyroid autoantibodies between 20% and 30% in CHC patients, the rate of 14.9% found for the CHC group in our study may be accepted as similar.

When the association of TD development with IFN therapy is evaluated separately in patients with CHB and CHC, statistically significant differences were not found in both groups (for CHB: $p = 0.33$, for CHC: $p = 0.58$). When evaluated with multiple logistic regression analysis, response status to IFN therapy was not found as a significant risk factor for TD development. While there are studies with similar results (12, 26), in the study by Tran *et al*. (27), a significant association was found between TD development and response to IFN therapy.

The main limitations of our study were it being a retrospective and uni-centre study, relatively low numbers of patients in CHB and CHC groups, and lack of investigation of some factors (viral load, degree of hepatic fibrosis, body mass index, tests of liver function) that could have been effective in TD development. In spite of these limitations, we believe that our results provide important information on the incidence of TD developing during IFN therapy in a region where CHB-C is commonly seen in Turkey, and on follow-up results of these patients. Our study also contributes to the literature, both globally and in Turkey.

CONCLUSION

According to our study, TD secondary to IFN therapy may develop in a substantial frequency (21.5%) in patients with CHB-C. Independent risk factors in TD development were found to be female sex and age (over 40 years). TD commonly develops in the first six

months and in the sub-clinic form, not requiring treatment. Prospective, multi-centre studies are needed on this issue in our country on larger patient samples.

REFERENCES

1. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr et al. Peginterferon alfa-2a plus ribavirin for chronic hepa¬titis C virus infection. N Engl J Med 2002; **347:** 975–82.
2. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001; **358:** 958–65.
3. Sokal EM, Bourgois A, Stephenne X, Silveira T, Porta G, Gardo¬vska D et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection in children and adolescents. J Hepatol 2010; **52:** 827–31.
4. Parana R, Cruz M, Santos-Jesus R, Ferreira K, Codes L, Cruz T. Thy¬roid disease in HCV carriers undergoing antiviral therapy with interferon plus ribavirin. Braz J Infect Dis 2000; **4:** 284–90.
5. Kryczka W, Brojer E, Kowalska A, Zarebska-Michaluk D. Thyroid gland dysfunctions during antiviral therapy of chronic hepatitis C. Med Sci Monit 2001; **7:** 221–5.
6. Carella C, Mazziotti G, Morisco F, Rotondi M, Cioffi M, Tuccillo C et al. The addition of ribavirin to interferon-alpha therapy in pa¬tients with hepatitis C virus-related chronic hepatitis does not modify the thyroid autoantibody pattern but increases the risk of developing hypothyroidism. Eur J Endocrinol 2002; **146:** 743–9.
7. Yan Z, Fan K, Fan Y, Wang X, Mao Q, Deng G et al. Thyroid dysfunction in Chinese patients with chronic hepatitis C treated with interferon alpha: incidence, long-term outcome and predictive factors. Hepat Mon 2012; **12:** e6390.
8. Fernandez-Soto L, Gonzalez A, Escobar-Jimenez F, Vazquez R, Ocete E, Olea N et al. Increased risk of autoimmune thyroid dis¬ease in hepatitis C vs hepatitis B before, during, and after discon¬tinuing interferon therapy. Arch Intern Med 1998; 158: 1445–8.
9. Tran HA, Attia JR, Jones TL, Batey RG. Pegylated inter¬feron-alpha2beta in combination with ribavirin does not aggravate thyroid dysfunction in comparison to regular interferon-alpha2beta in a hepatitis C population: meta-analysis. J Gastroenterol Hepatol 2007; **22:** 472–6.
10. Jamil KM, Leedman PJ, Kontorinis N, Tarquinio L, Nazareth S, McInerney M et al. Interferon-induced thyroid dysfunction in chronic hepatitis C. J Gastroenterol Hepatol 2009; **24:** 1017–23.
11. Costelloe SJ, Wassef N, Schulz J, Vaghijiani T, Morris C, Whiting S et al. Thyroid dysfunction in a UK hepatitis C population treated with interferon-alpha and ribavirin combination therapy. Clin Endocrinol (Oxf) 2010; **73:** 249–56.
12. Barut S, Gunal O, Erkorkmaz U, Yildiz F. Thyroid dysfunction in Turkish patients with chronic hepatitis C receiving peginterferon plus ribavirin in the period of 2005–2010. Braz J Infect Dis 2012; **16:** 448–51.
13. Prummel MF, Laurberg P. Interferon-alpha and autoimmune thy¬roid disease. Thyroid 2003; **13:** 547–51.
14. Andrade LJ, Atta AM, D'Almeida Junior A, Paraná R. Thyroid dysfunction in hepatitis C individuals treated with interferon-alpha and ribavirin —a review. Braz J Infect Dis 2008; **12:** 144–8.
15. Indolfi G, Stagi S, Bartolini E, Salti R, de Martino M, Azzari C et al. Thyroid function and anti-thyroid autoantibodies in untreated children with vertically acquired chronic hepatitis C virus infection. Clin Endocrinol (Oxf) 2008; **68:** 117–21.
16. Kabbaj N, Guedira MM, El Atmani H, El Alaoui M, Mohammadi M, Benabed K et al. Thyroid disorders during interferon alpha therapy in 625 patients with chronic hepatitis C: a prospective cohort study. Ann Endocrinol 2006; **67:** 343–7.
17. Themistoklis V, Panagiotis A, Georgios N, Konstantinos S, Kaliopi P, Nikolaos G et al. Thyroid dysfunction and long-term outcome during and after interferon-alpha therapy in patients with chronic hepatitis C Ann Acad Med 2011; **40(9):** 394–400.
18. Hsieh MC, Yu ML, Chuang WL, Shin SJ, Dai CY, Chen SC et al. Vi¬rologic factors related to interferon-alpha-induced thyroid dys¬function in patients with chronic hepatitis C. Eur J Endocrinol 2000; **142:** 431–7.
19. Hwang Y, Kim W, Kwon SY, Yu HM, Kim JH, Choe WH. Incidence of and risk factors for thyroid dysfunction during peginterferon αand ribavirin treatment in patients with chronic hepatitis C. Korean J Intern Med 2015; **30:** 792–800.
20. Amir Z, Fatemeh E, Majid S. Thyroid Dysfunction in Patients with Chronic Viral Hepatitis B and C during Alpha Interferon Therapy. Hepat Mon 2009; **9:** 110–3.
21. Preziati D, La Rosa L, Covini G, Marcelli R, Rescalli S, Persani L et al. Autoimmunity and thyroid function in patients with chronic active hepatitis treated with recombinant interferon alpha-2a. Eur J Endocrinol 1995; **132:** 587–93.
22. Dzekova-Vidimliski P, Nikolov I, Matevska-Geshkovska N, Boyanova Y, Nikolova N, Romanciuc G et al. Genetic predictors of the response to the treatment of hepatitis C virus infection. Bosn J Basic Med Sci 2015; **15:** 55–9.
23. Pavan MH, Pavin EJ, Goncales FL, Jr., Wittmann DE. Virus C gen¬otype predisposes to primary hypothyroidism during inter¬feron-alpha treatment for chronic hepatitis C. Braz J Infect Dis 2011; **15:** 449–56.
24. Huang JF, Chuang WL, Dai CY, Chen SC, Lin ZY, Lee LP et al. The role of thyroid autoantibodies in the development of thyroid dysfunc¬tion in Taiwanese chronic hepatitis C patients with in¬terferon-alpha and ribavirin combination therapy. J Viral Hepat 2006; **13:** 396–401.
25. Vasiliadis T, Anagnostis P, Nalmpantidis G, Soufleris K, Patsiaoura K, Grammatikos N et al. Thyroid dysfunction and long-term outcome during and after interferon-alpha therapy in patients with chronic hepati¬tis C. Ann Acad Med 2011; **40:** 394–400.
26. Dalgard O, Bjøro K, Hellum K, Myrvang B, Bjøro T, Haug E et al. Thyroid dysfunction during treatment of chronic hepatitis C with interferon alpha: no association with either interferon dosage or efficacy of therapy. J Intern Med. 2002; **251:** 400–6.
27. Tran HA, Malcolm Reeves GE, Gibson R, Attia JR. Development of thyroid diseases in the treatment of chronic hepatitis C with alpha-interferon may be a good prognosticator in achieving a sustained virological response: a meta-analysis. J Gastroenterol Hepatol 2009; **24:** 1163–8.

ORIGINAL ARTICLE

Evaluation of the Antioxidant Properties, Fatty Acid Profile, Ash, Zinc, Crude Fibre and Pectin Content of Jamaican Sorrel (*Hibiscus sabdariffa*) and Citrus (*Citrus sinensis*) By-products from a Local Beverage Manufacturer

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ABSTRACT

Objective: *Sorrel* (Hibiscus sabdariffa) *and citrus* (Citrus sinensis) *are widely utilised in the local beverage industry. By-products from this industry are mainly utilised as fodder and fertiliser but may be of commercial value. Phytochemical analyses were therefore performed on extracts from by-products of this industry. Extracts were evaluated for their antioxidant activity, total phenolics, fatty acid, crude fibre, pectin, ash and zinc content.*

Methods: *Antioxidant activity and total phenolics were evaluated by a spectrophotometric assay. Lipids were Soxhlet extracted and characterised by nuclear magnetic resonance spectroscopy and gas chromatography/mass spectrometry. Zinc content was determined by atomic absorption spectroscopy. Crude fibre, pectin and ash were determined gravimetrically.*

Results: *Extracts showed antioxidant activity with methanolic extracts containing higher levels of phenolics than ethyl acetate extracts. Lipids were a source of omega 6 and omega 9 fatty acids. Palmitoleic acid was also identified in citrus extracts. Palmitic acid is the major saturated fatty acid present. Based on the predicted iodine value, citrus lipid extracts are expected to be more stable to oxidation compared to sorrel extracts. Zinc analysis by atomic absorption spectroscopy revealed that sorrel extracts had higher levels of zinc (367.3 ppm) compared to citrus extracts (Zn 5.0 ppm). Extracts were a minor source of pectin and crude fibre.*

Conclusion: *By-products from the local beverage industry may be considered for the use in food and nutraceutical applications. They are a source of antioxidants, essential fatty acids, zinc and crude fibre.*

Keywords: Antioxidant activity, *Citrus sinensis*, fatty acid, *Hibiscus sabdariffa*, phenolics, zinc

INTRODUCTION

Hibiscus sabdariffa is an annual herb or shrub popularly known as roselle or sorrel belonging to the Malvaceae family. It is native to India and Malaysia, where it is cultivated. The plant is also grown in Jamaica, South America and Africa. The calyx of *H. sabdariffa* is widely used as food, in jams, jellies, pickles, juice drinks, wine and as medicinal syrups (1). In Jamaica and some countries in Africa, the sorrel calyx is used to produce a traditional drink. In Nigeria, the drink is referred to as 'zoborodo'. The intense red colour of the calyx is due to the presence of anthocyanins. The major anthocyanins present are delphinidin 3-sambubioside and cyanidin 3-sambubioside (2). In addition to anthocyanins, other components present include alkaloids, flavonoids, saponins, steroids, sterols and tannins (2). Sorrel has powerful antioxidant properties (3), which helps lower elevated blood pressure, bad cholesterol and detoxifies the body. Sorrel extracts have also been reported as possessing antimicrobial activity and is active against *E. coli* 0157:H7, a significant cause of foodborne and waterborne disease (4).

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Citrus sinensis is widely cultivated and is eaten fresh or processed into jams, juices and marmalades. It belongs to the family Rutaceae. The peel of the fruit is rich in flavonoids, alkaloids, saponins, tannins, triterpenoids, phytosterols and steroids, possessing medicinal properties such as anti-viral, anti-bacterial and anti-oxidant activities (5, 6). The fruit is well known for its ascorbic acid content and antioxidant activity (7).

H. sabdariffa and *C. sinensis* are utilised in Jamaica's local beverage industry. This industry produces a considerable quantity of solid and liquid residue, rich in soluble sugars, cellulose, hemicellulose, pectin and essential oils, which is considered as waste (8). The waste in most cases is spread on soil in areas adjacent to the production location, for its final use as a raw material in animal feed (9). Alternatively, it may be burned. This method of handling waste produces highly polluted wastewater with high chemical and biological oxygen demands, which can negatively affect the soil, the ground and superficial waters (10).

An alternative is to improve the management of these residues, which may be used as a substrate for the production of value-added compounds or in the production of organic fertilisers, pectin, bio-oils, essential oils and antioxidant compounds. These alternatives avoid the environmental pollution and add the value to these substances. Phytochemical analyses were therefore conducted on by-products from the Jamaica's sorrel and citrus beverage industries.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade. The Folin-Ciocalteu reagent and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich (USA).

Plant material

Sorrel calyces and citrus (peel, pulp and seeds) by-products were obtained from a local juice processing plant in St Catherine, Jamaica. Samples were air dried at ambient temperature (four days) and milled.

The DPPH radical-scavenging assay

The DPPH assay was performed according to the method of Brand-Williams *et al.* (11). Concentrates were dissolved in methanol (1:3) and then reacted (1 mL) with DPPH (0.004%, 1 mL, 30 minutes). The absorbance was measured at 517 nm using a spectrophotometer (Thermo Fisher Scientific). A standard calibration curve was generated, and the results were expressed as µmol/g (gallic acid equivalents).

Total phenolic content assay

The total phenolic content was determined by the Folin-Ciocalteu method, adopted and modified from Swain and Hillis (12). The samples were extracted with hexane, ethyl acetate and methanol. The extracts (500 µL) were reacted with the Folin-Ciocalteu reagent (10%, 2.5 mL) and Na_2CO_3 solution (7.5%, 2 mL). The solution was incubated at room temperature in the dark (30 minutes) and the absorbance measured at 760 nm using a spectrophotometer (Thermo Fisher Scientific). A standard calibration curve was generated, and the results were expressed as mg gallic acid equivalent/g.

Lipid extraction

Sorrel and citrus by-products were dried to constant weight (60°C, 8 hours) and milled. The samples were lipid-extracted with petroleum ether (bp: 80°C–100°C, reflux), using a Soxhlet extractor. The resulting extract was concentrated *in vacuo* and the percent crude fat was determined gravimetrically.

Methylation of lipid extracts

The lipid extracts (50 µL) were *trans*-methylated with the methanol/acetyl chloride solution (13). The resulting fatty acid methyl esters (FAMEs) were determined by the gas chromatography-mass spectrometry (GC-MS).

Gas chromatography-mass spectrometry

The methylated samples (1.0 µL) were chromatographed on a HP6890 series gas chromatograph interfaced with a HP5973 Mass Selective Detector. The constituent FAMEs were eluted in helium carrier gas (flow rate: 1 cm^3 per minute) through a DB-VRX column (20 m × 0.18 mm i.d. × 1.0-µm-film thickness, Agilent, CA, USA) in an oven programmed at 60°C for 3 minutes and increased at a ramp rate of 10°C/minutes up to 250°C for 15 minutes. The samples were injected at 230°C, although the detector was maintained at 250°C. The constituents were identified with the National Institute of Standards and Technology (NIST) library of mass spectra and subsets (match quality > 80%).

^{1}H-NMR and ^{13}C-NMR spectroscopy

^{1}H-NMR and ^{13}C-NMR characterisation were performed on a Bruker BioSpin 200 MHz at 200 MHz. The samples were run in the deuterated chloroform ($CDCl_3$) at 25°C, with tetra-methylsilane as the internal standard.

In reporting ^{1}H-NMR, the following terms were used: singlet (s), doublet (d), triplet (t) and multiplet (m).

Iodine value

Iodine values were determined based on the FAME content and was calculated utilising the formula:

$$\text{Predicted IV} = xC1 + yC2 + zC3$$

C1, C2 and C3 correspond to the relative percentage concentrations of unsaturated fatty acids (one, two and three double bonds, respectively), whereas x, y and z are coefficients (x = 1, y = 1.5 and z = 2.62) (14).

Atomic absorption spectroscopy

The samples (1.5 g) were ashed in a muffle furnace (600°C for 1.5 hours) and the resulting ash was dissolved in HCl (10 ml, AR), diluted with deionised water and filtered. Measurements for zinc were made using a Perkin Elmer 2380 Flame Atomic Absorption Spectrophotometer system equipped with a zinc-hollow cathode lamp. The following parameters were utilised: lamp current 10 mA, wavelength 214 nm, slit width 0.7 nm with flame type consisting of air/acetylene and stoichiometric fuel flow at 0.9–1.21 per minute. A stock solution of zinc was made, and a standard calibration curve was prepared. The results were expressed as ppm.

Crude fibre

The samples (12 g) were weighed and sulphuric acid (0.1 M) was added. The mixture was boiled (30 minutes) and then filtered. The resulting residue was rinsed with distilled water. Sodium hydroxide (0.3 M) was added to the residue, which was followed by boiling (30 minutes) and filtration. The residue was washed with water, hydrochloric acid (1%) and ethanol. The insoluble matter was then transferred to a crucible and allowed to dry (100°C) after which samples were ashed (550°C, 2 hours) and the crude fibre content was determined gravimetrically.

Pectin content

The samples (50 g) were suspended in de-ionised water (400 mL) and boiled (1 hour). The extracts were filtered and sodium hydroxide (1 M) was added. The solution was allowed to stand (24 hours) after which acetic acid (1 M) was added followed by calcium chloride (0.5 M). The solution was then boiled and filtered. The resulting residue was dried (105°C, 3 hours) and the mass of pectin was determined gravimetrically.

RESULTS AND DISCUSSION

Antioxidant activity and total phenolics

Free radicals are produced in the cells continually forming an integral part of cellular function. Over production may lead to oxidative stress, which can result in the development of chronic and degenerative diseases such as cancer, neurological disorders and rheumatoid arthritis (15). The antioxidants assist in preventing cell damage by limiting the formation of free radicals, acting as free radical scavengers and promoting their decomposition (16). *H. sabdariffa* and *C. sinensis* have gained attention for their antioxidant activity (17–19). The sorrel and citrus by-product extracts possessed similar antioxidant activities (141.57 ± 5.27 µmol/g and 139.77 ± 4.81 µmol/g gallic acid equivalents, respectively) and may be utilised as a source of natural antioxidants. Flavones and glycosylated flavanones are the main phenolics, contributing to the antioxidant activity of citrus extracts (19). Phenolics and anthocyanins are primarily responsible for the antioxidant activity of sorrel extracts (20). The citrus extracts contained higher levels of phenolics compared to the sorrel extracts (Table 1). A study on 13 citrus species revealed that total phenolics was higher in the peel of the fruit (21). Naringin and neohesperidin have been identified as the major polyphenols in the methanolic extracts of bitter orange peels; narirutin and hesperidin in sweet orange peels (22). The total phenolics in different citrus species ranges from 66.5 to 396.8 mg gallic acid equivalent/g (21), although sorrel has been reported as containing a total phenolic content of 41.07 mg gallic acid equivalent/g (23).

Table 1: Total phenolic content of citrus and sorrel by-product extracts expressed as gallic acid equivalents (GAE)

Extraction solvent	Citrus extract (mg/g)	Sorrel extract (mg/g)
Hexane	ND	8.08 ± 1.96
Ethyl acetate	63.08 ± 8.53	21.68 ± 4.62
Methanol	118.09 ± 6.98	33.14 ± 2.38

ND = not detected.

Lipid analysis

Citrus oil concentrates have been utilised commercially in a wide number of applications. These include their use for flavour and fragrances. D-Limonene has been identified as the major component present in the essential oil extracts of citrus (24). Less reported is the utilisation of sorrel oil extracts. Sorrel seed oil (25) which is rich in linoleic acid and oleic acid has been investigated for its nutritional properties (26) and its potential as a source of biofuel (27). The oil is also a good source of

the lipid-soluble antioxidant gamma tocopherol (28). Currently, there is no literature on the fatty acid composition of sorrel calyces. The lipid content of the sorrel and citrus by-product extracts were 3.15% ± 1.22% and 3.70% ± 1.30%, respectively. Linoleic acid and oleic acid were identified as the major unsaturated fatty acids and palmitic acid was identified as the major saturated fatty acid (Table 2). Palmitoleic acid (omega 7) was identified in small quantities in citrus by-product extracts. Macadamia oil is a good source of palmitoleic acid (29). Palmitoleic acid has shown lipid lowering and anti-inflammatory benefits and may be useful in the treatment of hyper-triglyceridemia (30). The predicted iodine value, an indicator of the oil's stability to oxidation, was higher in the sorrel extracts compared to the citrus extracts (59; 49, respectively) due to the higher levels of linoleic acid and oleic acid present. The citrus lipid extracts are therefore expected to be more stable to the oxidative rancidity.

Table 2: Fatty acid profile of sorrel and citrus by-product extracts

Fatty acid		Sorrel (%)	Citrus (%)
Palmitic acid	C16:0	42.45 ± 14.35	32.94 ± 7.40
Palmitoleic acid (omega 7)	C16:1	ND	0.74 ± 0.07
Stearic acid	C18:0	7.64 ± 2.32	7.21 ± 1.49
Oleic acid (omega 9)	C18:1	20.82 ± 2.95	18.24 ± 0.80
Linoleic acid (omega 6)	C18:2	25.37 ± 6.33	19.90 ± 12.79

ND = not detected.

^{1}H- and ^{13}C-NMR spectroscopy

The lipid extracts are comprised predominantly of triacylglycerols. ^{1}H-NMR spectroscopy of the crude citrus and sorrel lipid extracts revealed the presence of two doublet of doublets (δ 4.14–4.30) and a multiplet (δ 5.34), which are consistent with the protons of the glyceryl moiety of a triacylglycerol (Table 3). The *bis*-allylic protons due to linoleic acid resonated as a singlet at δ 2.76. Olefinic protons were observed at δ 5.37. ^{13}C-NMR data confirmed the presence of the glycerol backbone of the triacylglycerols with signals resonating at δ 62.0 ppm (α) and δ 68.9 ppm (β). Unsaturation within fatty acid side chains due to the presence of linoleic acid and oleic acid was observed in the range of δ 129–130 ppm (31).

Ash

Ash is the inorganic matter remaining after heating organic components in the presence of oxidizing agents. It indicates total mineral content. The citrus and sorrel samples contained 3.2% ± 0.4% and 4.4% ± 0.3% ash, respectively. Values of 1.5% (32) and 4.8% (33) have been reported for orange peels and 6.5% (34) and 12.2% (35) for sorrel calyces from Nigeria. Higher percentage ash values are seen in edible wild plants from Turkey, with values ranging from 7.5% to 18.5% for *Tragopogon aureus* Boiss and *Urtica dioica* L., respectively (36). Values of 1.03% and 0.51% have been reported for *Daucus carota* and *Lycopersicom esculentum*, respectively (37).

Table 3: ^{1}H-nuclear magnetic resonance spectroscopy data of sorrel and citrus by-product extracts

Proton	Functionality	Sorrel (δ ppm)	Citrus (δ ppm)
CH_3	Terminal methyl	0.86 (m)	0.88 (m)
CH_2	Methylene	1.26 (s)	1.25 (s)
CH_2–CH_2–COO	All acyl chains	1.61 (m)	1.60 (m)
CH_2–CH=CH	All unsaturated fatty acids	2.05 (s)	2.02 (m)
CH_2–COO	All acyl chains	2.28 (s)	
		2.35 (m)	2.34 (t)
C=C–CH_2=C	Protons attached to bisallylic carbon	2.76 (s)	2.76 (s)
$CH_2O(\alpha)$	Glycerol (triglycerides)	4.14 (dd)	4.15 (dd)
		4.26 (dd)	4.30 (dd)
CHO(β)	Glycerol (triglycerides)	5.34 (m)	5.34 (m)
CH=CH	Olefinic protons	5.37 (m)	5.37 (m)
COOH	Free fatty acids	7.27 (s)	7.27 (s)

m = multiplet; s = singlet; dd = doublet of doublet; t = triplet.

Zinc content

Zinc is an essential trace element, which is necessary for the human health and well-being. The role of zinc in the body is both structural and functional. It plays an important role in over 300 enzymatic reactions participating in different aspects of intermediary metabolism (38). High amount of zinc can be found in the brain. Metalloproteins containing zinc serve as the neuromodulators and neurotransmitters (39). Severe zinc deficiency can lead to behavioural problems and mood disorders (40). Zinc is required for the normal immune function (38), possesses antioxidant (41) and anti-inflammatory properties (42). The ability of zinc to retard oxidative processes has been recognised for several years (43). The mechanism is believed to be by protection of sulfhydryl groups against oxidation and inhibition of the production of reactive oxygen by transition metals (44). Zinc also plays a structural role in the bone matrix (38). Bone mineral is composed of hydroxyapatite crystals, which contains zinc complexed with fluoride (38). The sorrel extracts investigated contained higher concentrations of zinc (367.3 ppm) compared to citrus (5.0 ppm) and may be considered a good source of zinc.

Crude fibre

Plants are a source of dietary fibre that plays an important role in decreasing the risk of various disorders such as diabetes, cardiovascular disease, diverticulosis and obesity (45). Dietary fibre may be classified as water soluble/less fermentable (cellulose, hemicellulose and lignin) or water insoluble/fermentable (pectin, gums and mucilages) (46) and is utilised in various foods such as baked products, beverages and meat products. The use of fibre in foods not only enhances its health benefits but also leads to changes in the sensorial and rheological properties of the food. The crude fibre which consists mainly of cellulose, hemicellulose and lignin was determined by the sequential extraction with acid and base followed by ashing. The crude fibre contents of sorrel and citrus by-products were (4.16% ± 0.36% and 3.94% ± 0.32%), respectively. Sorrel calyces from Nigeria have been reported as containing 8.5% crude fibre (34) and 2.7% orange peels (33). When compared with other fruits, the crude fibre content of citrus and sorrel by-products is higher than that reported for watermelon (0.2%), mango (0.7%) and pomegranate (2.1%) but comparable to custard apple (3.1%), dates (3.7%) and guava (5.2%) (47). The nutritional benefits of food fibres have led to the development of fibre-rich products. The alternative and novel sources of dietary fibre continue to be investigated for the use in food supplementation. Non-traditional sources such as by-products from food processing continue to be evaluated, for example, spent brewer's grain, seed hulls and corn stalks (48). Nassar *et al.* (49) suggested that 15% of orange peel and pulp could be incorporated as an ingredient in the baked products, as they are a source of dietary fibre with bioactive components.

Pectin

Pectin is a major component of the primary cell walls of plants and encompasses a range of galacturonic acid-rich polysaccharides (50). The major neutral sugars of hydrolysed pectin are arabinose, galactose and rhamnose with galacturonic acid contents ranging from 63% to 64% (51). The Food and Agriculture Organization stipulates that pectin must contain at least 65% galacturonic acid. Pectin is widely used in the food industry due to its ability to form gels as well as the cosmetic and pharmaceutical industries. Commercial sources of pectin are traditionally from apple pomace and citrus peel. Recent research has identified passion fruit peels as containing high levels of pectin with yields of 10%–20% being reported (52, 53). The pectin extraction is normally performed with a diluted strong mineral acid solution or weak organic acids, for example, citric acid (52, 54, 55). Sorrel and citrus by-products from the current study were extracted with acetic acid. Small quantities of pectin (sorrel, 0.48% ± 0.13% and citrus, 0.84% ± 0.10%) were recovered from the samples investigated. This may be due to pectin losses during the juice extraction process. Improved extraction efficiencies have been reported with the use of ammonium oxalate. Pectin extracted from sorrel utilising ammonium oxalate gave yields of 18.7% compared with hydrochloric extractions (9.8%) (56). pH is also an important parameter in the extraction of pectin. Maximum pectin yields of 14.6% were obtained from passion fruit peel at a pH of 2. Orange peels that contain 1.7% pectin on a dry weight basis and 15.9% on a wet weight basis (57) are traditionally used as a source of pectin.

Relevance and potential applications

By-products from the sorrel and citrus industry may be considered for the use in nutraceuticals, food fortification and an ingredient of functional foods. They are a source of antioxidants, omega fatty acids, zinc and crude fibre. Sorrel calyx by-products could be utilised in fruit snack formulations and chutneys. In recent years, there has been a growing interest in natural antioxidants and colourants with consumers, showing the increased concerns over the use of chemically synthesised dyes and artificial antioxidants due to their suspected carcinogenic effects.

CONCLUSION

Agro-processing by-products can be better managed and utilised for various commercial applications. The conversion of cellulosic biomass to useable product presents an opportunity to reduce the greenhouse gas emission, reduce the global warming and utilise the by-products arising from the food industry.

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REFERENCES

1. Cid-Ortega S, Guerrero-Beltrán JA. Roselle calyces (*Hibiscus sabdariffa*), an alternative to the food and beverages industries: a review. J Food Sci Technol 2015; **52:** 6859–69.
2. Obouayeba AP, Djyh NB, Diabate S, Djaman AJ, N'Guessan JD, Kone M et al. Phytochemical and antioxidant activity of roselle (*Hibiscus sabdariffa* L.) petal extracts. Res J Pharm Biol Chem Sci 2014; **5:** 1453–65.

3. Mutai E, Sunkara R, Vizcarra J, Walker LT, Verghese M. Antioxidant, enzyme inhibitory and anti-obesity potential of sorrel calyx extracts in 3T3-L1 adipocytes. Food Nutr Sci 2015; **6:** 452–65.
4. Fullerton M, Khatiwada J, Johnson JU, Davis S, Williams LL. Determination of antimicrobial activity of sorrel (*Hibiscus sabdariffa*) on Escherichia coli O157:H7 isolated from food, veterinary, and clinical samples. J Med Food 2011; **14:** 950–6.
5. Lawal D, Bala JA, Aliyu SY, Huguma MA. Phytochemical screening and in vitro anti-bacterial studies of the ethanolic extract of *Citrus sinensis* (Linn.) peel against some clinical bacterial isolates. IJIAS 2013; **2:** 138–45.
6. Hegazy AE, Ibrahium MI. Antioxidant activities of orange peel extracts. World Appl Sci J 2012; **18:** 684–8.
7. Chatha SAS, Hussain AI, Asi MR, Majeed M, Iqbal HMN. Evaluation of antioxidant potential of citrus peel extracts. J Chem Soc Pak 2011; **33:** 863–8.
8. Rezzadori K, Benedetti S, Amante ER. Proposals for the residues recovery: orange waste as raw material for new products. Food Bioprod Process 2012; **90:** 606–14.
9. Martin MA, Siles JA, Chica AF, Martín A. Biomethanization of orange peel waste. Bioresour Technol 2010; **101:** 8993–9.
10. Braddock RJ. By-products of citrus fruit. Food Technol 1995; **49:** 74–7.
11. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci Technol 1995; **28:** 25–30.
12. Swain T, Hillis WE. The phenolic constituents of Prunus domestica. I. The quantitative analysis of phenolic constituents. J Sci Food Agr 1959; **10:** 63–8.
13. Masood A, Stark KD, Salem N Jr. A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. J Lipid Res 2005; **46:** 2299–305.
14. Kyriakidis NB, Katsiloulis T. Calculation of iodine value from measurements of fatty acid methyl esters of some oils: comparison with the relevant American Oil Chemists Society method. J Am Oil Chem Soc 2000; **77:** 1235–8.
15. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci 2008; **4:** 89–96.
16. Young IS, Woodside JV. Antioxidants in health and disease. J Clin Pathol 2001; **54:** 176–86.
17. Christian KR, Nair MG, Jackson JC. Antioxidant and cyclooxygenase inhibitory activity of sorrel (*Hibiscus sabdariffa*). J Food Compost Anal 2006; **19:** 778–83.
18. Christian KR, Jackson JC. Changes in total phenolic and monomeric anthocyanin composition and antioxidant activity of three varieties of sorrel (*Hibiscus sabdariffa*) during maturity. J Food Compost Anal 2009; **22:** 663–7.
19. Bocco A, Cuvelier ME, Richard H, Berset C. Antioxidant activity and phenolic composition of citrus peel and seed extracts. J Agr Food Chem 1998; **46:** 2123–9.
20. Yang L, Gou Y, Zhao T, Zhao J, Li F, Zhang B et al. Antioxidant capacity of extracts from calyx fruits of roselle (*Hibiscus sabdariffa* L.). Afr J Biotechnol 2012; **11:** 4063–8.
21. Ghasemi K, Ghasemi Y, Ebrahimzadeh MA. Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. Pak J Pharm Sci 2009; **22:** 277–81.
22. Sawalha SMS, Arráez-Román D, Segura-Carretero A, Fernández-Gutiérrez A. Quantification of main phenolic compounds in sweet and bitter orange peel using CE–MS/MS. Food Chem 2009; **116:** 567–74.
23. Sirag N, Elhadi MM, Algaili AM, Hassan HM, Ohaj M. Determination of total phenolic content and antioxidant activity of Roselle (*Hibiscus sabdariffa* L.) calyx ethanolic extract. Stand Res J Pharm 2014; **1:** 34–9.
24. Darjazi BB. Comparison of peel oil components of grapefruit and lime (Citrus sp.). Intl J Agri Crop Sci 2013; **6:** 840–7.
25. Betiku E, Adepoju TF. Sorrel (*Hibiscus sabdariffa*) seed oil extraction optimization and quality characterization. Am Chem Sci J 2013; **3:** 449–58.
26. Samy MS. Chemical and nutritional studies on roselle seeds (*Hibiscus sabdariffa* L.). Z Ernahrungswiss 1980; **19:** 47–9.
27. Nakpong P, Wootthikanokkhan S. Roselle (*Hibiscus sabdariffa* L.) oil as an alternative feedstock for biodiesel production in Thailand. Fuel 2010; **89:** 1806–11.
28. Mohamed R, Fernández J, Pineda M, Aguilar M. Roselle (*Hibiscus sabdariffa*) seed oil is a rich source of gamma-tocopherol. J Food Sci 2007; **72:** S207–11.
29. Garg ML, Blake RJ, Wills RBH, Clayton EH. Macadamia nut consumption modulates favourably risk factors for coronary artery disease in hypercholesterolemic subjects. Lipids 2007; **42:** 583–7.
30. Bernstein AM, Roizen MF, Martinez L. Purified palmitoleic acid for the reduction of high-sensitivity C-reactive protein and serum lipids: a double-blinded, randomized, placebo controlled study. J Clin Lipidol 2014; **8:** 612–17.
31. Thoss V, Murphy PJ, Marriott R, Wilson T. Triacylglycerol composition of British bluebell (Hyacinthoides non-scripta) seed oil. RSC Adv 2012; **2:** 5314–22.
32. Ververis C, Georghiou K, Danielidis D, Hatzinikolaou DG, Santas P, Santas R et al. Cellulose, hemicelluloses, lignin and ash content of some organic materials and their suitability for use as paper pulp supplements. Bioresour Technol 2007; **98:** 296–301.
33. Azam S, Khan Z, Ahmad B, Khan I, Ali J. Production of single cell protein from orange peels using Aspergillus niger and *Saccharomyces cerevisiae*. Global J Biotechnol Biochem 2014; **9:** 14–18.
34. Babalola SO, Babalola AO, Aworh OC. Compositional attributes of the calyces of roselle (*Hibiscus sabdariffa* L.). J Food Technol Afr 2001; **6:** 133–4.
35. Adanlawo IG, Ajibade VA. Nutritive value of the two varieties of roselle (*Hibiscus sabdariffa*) calyces soaked with wood ash. Pak J Nutr 2006; **5:** 555–7.
36. Cakilcioglu U, Khatun S. Nitrate, moisture and ash contents of edible wild plants. J Cell Plant Sci 2011; **2:** 1–5.
37. Motegaonkar MB, Salunke SD. The ash and iron content of common vegetable grown in Latur District, India. Res J Recent Sci 2012; **1:** 60–3.
38. Meunier N, O'Connor JM, Maiani G, Cashman KD, Secker DL, Ferry M et al. Importance of zinc in the elderly: the ZENITH study. Eur J Clin Nutr 2005; **59:** S1–4.
39. Harrison NL, Gibbsons SJ. Zn^{2+}: an endogenous modulator of ligand- and voltage-gated ion channels. Neuropharmacol 1994; **33:** 935–52.
40. McLoughlin IJ, Hodge JS. Zn in depressive disorder. Acta Psychiatr Scand 1990; **82:** 451–3.
41. Bray TM, Bettger WJ. The physiological role of zinc as an antioxidant. Free Radical Biol Med 1990; **8:** 281–91.
42. Prasad AS. Zinc: an antioxidant and anti-inflammatory agent: role of zinc in degenerative disorders of aging. J Trace Elem Med Biol 2014; **28:** 364–71.
43. Powell SR. The antioxidant properties of zinc. J Nutr 2000; **130:** 1447S–54S.
44. Bettger WJ. Zinc and selenium, site specific vs general antioxidant. Can J Physiol Pharmacol 1993; **71:** 721–4.
45. Lattimer JM, Haub MD. Effects of dietary fiber and its components on metabolic health. Nutrients 2010; **2:** 1266–89.
46. Dhingra D, Michael M, Rajput H, Patil RT. Dietary fibre in foods: a review. J Food Sci Technol 2012; **49:** 255–66.
47. Ramulu P, Rao PU. Total, insoluble and soluble dietary fiber contents of Indian fruits. J Food Compost Anal 2003; **16:** 677–85.
48. Katz F. Putting the function in functional food. Food Process 1996; **57:** 56–8.
49. Nassar AG, AbdEl-Hamied AA, El-Naggar EA. Effect of citrus by-products flour incorporation on chemical, rheological and organoleptic characteristics of biscuits. World J Agric Sci 2008; **4:** 612–16.
50. Willats WG, McCartney L, Mackie W, Knox JP. Pectin: cell biology and prospects for functional analysis. Plant Mol Biol 2001; **47:** 9–27.
51. Day A, Dasgupta PC, Sardar D. Pectic substances from mesta (Hibiscus cannabinus) and roselle (*Hibiscus sabdariffa*) plants. Curr Sci 1983; **52:** 885–6.
52. Pinheiro ER, Silva IMDA, Gonzaga LV, Amante ER, Teófilo RF, Ferreira MMC et al. Optimization of extraction of high-ester pectin from passion fruit peel (Passiflora edulis Flavicarpa) with citric acid by using response surface methodology. Bioresour Technol 2008; **99:** 5561–6.

53. Seixas FL, Fukuda DL, Turbiani FRB, Garcia PS, Petkowicz CLO, Jagadevan S et al. Extraction of pectin from passion fruit peel (Passiflora edulis F. Flavicarpa) by microwave-induced heating. Food Hydrocoll 2014; **38:** 186–92.
54. Minjares-Fuentas R, Femenia A, Garau MC, Meza-Velazquez JA, Simal S, Rossello C. Ultrasound-assisted extraction of pectins from grape pomace using citric acid: a response surface methodology approach. Carbohydr Polym 2014; **106:** 179–89.
55. Kulkarni SG, Vijayanand P. Effect of extraction conditions on the quality characteristics of pectin from passion fruit peel (Passiflora edulis f. flavicarpa L.). LWT-Food Sci Technol 2010; **43:** 1026–31.
56. Nazaruddin R, Noor Baiti AA, Foo SC, Tan YN, Ayob MK. Comparative chemical characteristics of hydrochloric acid- and ammonium oxalate-extracted pectin from roselle (*Hibiscus sabdariffa* L.) calyces. Int Food Res J 2013; **20:** 281–4.
57. Aina VO, Barau MM, Mamman OA, Zakari A, Haruna H, Hauwa Umar MS et al. Extraction and characterization of pectin from peels of lemon (*Citrus limon*), grape fruit (*Citrus paradisi*) and sweet orange (*Citrus sinensis*). Br J Clin Pharmacol 2012; **3:** 259–62.

ORIGINAL ARTICLE

The Urinary Levels of Some Essential and Toxic Metals in Type 2 Diabetic Patients and Non-diabetic Healthy Control Human Subjects

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ABSTRACT

Objective: *To compare the levels of some selected essential and toxic metals in type 2 diabetic (T2D) patients and non-diabetic (ND) healthy controls. Furthermore, the study aims to evaluate the possible interrelationship of metals in urine of T2D patients and ND healthy controls.*

Methods: *The study was conducted at the Diabetes and Cardio-Metabolic Disorder Laboratory, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan, which comprised T2D patients and an equal number of ND healthy controls. Two biofluids,* ie*, blood and urine samples of diabetes and control subjects were considered. Magnesium (Mg), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), manganese (Mn), iron (Fe) and zinc (Zn) levels were measured in the urine samples of both groups. Metal concentrations were assessed by atomic absorption spectrophotometry, whereas biochemical parameters in the serum of the study subjects were estimated using commercial kits on Microlab-300. Graph Pad Prism 5 was used for statistical analyses.*

Results: *There were 49 T2D patients with an average age of 53.5 ± 10.4 years and 49 ND healthy controls with a mean age of 45.2 ± 9.2 years. Fasting serum glucose and triglycerides were significantly (*p *< 0.05) higher in diabetics relative to their ND control subjects. The levels of Mg, Zn and Pb were found to be significantly higher (*p *< 0.05). Fe decreased more than 30% in urine samples of T2D patients compared with the ND control group. Cu, Cr, Mn and Ni manifested no change. A significant positive correlation of Pb with Zn (r = 0.3284,* p *= 0.02) and Mn (r = 0.3648,* p *= 0.01) was found in T2D patients.*

Conclusion: *An imbalance in the levels of selected metals was observed in urines of type 2 diabetes when compared with controls. Furthermore, statistical results revealed positive correlations between toxic metal Pb, with essential trace elements,* ie*, Mn and Zn. Thus, it could be concluded that toxic metals, especially Pb may have a role in renal dysfunction with successive loss of essential metals such as Zn and Mn through urination.*

Keywords: Essential metals, toxic metals, type 2 diabetes, urine.

INTRODUCTION

Diabetes mellitus (DM) is one of the lifestyle-related diseases that is the most widespread worldwide. Diabetes mellitus is usually classified as type 1 diabetes and type 2 diabetes (T2D). In the case of the former case, the hyperglycaemia results from a severe insulin deficiency caused by the destruction of pancreatic beta cells (1). Over the time, it can be regulated by using different

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insulin modalities (2, 3). In the latter case, glucose elevation develops because of insulin resistance (IR) and insufficient insulin secretion. Insulin is secreted from pancreatic beta cells in response to increased blood glucose levels. Insulin binds to its receptor on the surface of target tissues and stimulates glucose uptake by the cells. This result in blood glucose level being decreased. A decreased response to insulin may result in impaired glucose tolerance or T2D, which is characterised by elevated blood glucose level and IR (4). Several factors such as heredity, age, smoking, obesity, diet, sex, sedentary life style, socioeconomic status and hypertension, *etc*, are involved in the disease development. Among all these parameters, balanced diet and active lifestyle play vital roles in T2D prevention (5, 6).

The quality and quantity of fat and carbohydrates-based diet intake have significant impact on the risk of T2D (7). Imbalance in biologically active form of many metals such as iron (Fe), magnesium (Mg), zinc (Zn), chromium (Cr), manganese (Mn), lead (Pb), nickel (Ni), and copper (Cu), participate in the alteration of glucose metabolism of T2D patients (8). These metals are involved in the structural and catalytic activities of many metalloenzymes. Magnesium is needed as a cofactor for more than 300 enzymes, particularly involved in glucose homeostasis. The reduced plasma level of Mg has been reported in poorly controlled T2D patients (9, 10). Prolonged hyperglycaemia is also due to increased and decreased urinary and blood plasma levels of Zn. The decreased plasma level of Zn adversely affects the ability of pancreatic islet cells to produce and secrete insulin (11). Zinc plays a key role in the storage and secretion of insulin. Previous studies show that people with T2D have suboptimal Zn status due to its low absorption and increased urinary excretion (8). Copper is needed for the catalytic activity of superoxide dismutase that takes part in the protection of cells from superoxide radicals (8).

Whereas Zn protects sulfhydryl groups of proteins and enzymes by its antioxidant properties from free radical attack in the body, it is also reported that glycated proteins bind transition metals and their glycocholates play a key role in the aetiology of peripheral vascular dysfunction in T2D (12). Chromium is involved in glucose metabolism by enhancing the effects of insulin. Certain low molecular weight Cr (LMWCr) compounds may enhance the response of insulin receptors to insulin. The ability of LMWCr compounds to activate the insulin receptors depends upon its Cr contents. When the insulin level drops due to normalisation of blood glucose level, LMWCr compounds may be released from the cell in order to diminish its effects (13).

Iron is a potential candidate in many cellular reactions that produce reactive oxygen species (ROS). Such reactions contribute to tissue damage and increased oxidative stress, thereby leading to the pathogenesis of T2D. Numerous studies have suggested a link between high body iron stores and diabetes pathogenesis (14, 15). Similarly, Pb is dangerous to most of the human body organs and interferes with metabolism and cellular functions. Some other toxic metals such as cadmium and arsenic are implicated as they disrupt the glucose uptake and alter the related molecular mechanism in glucose regulation (16, 17).

The concentration of these metals in various biofluids is adversely changed with disease state, particularly in serum and urine. Among these biospecimens, urine is good since it is freely obtainable and non-invasively tested (8). Therefore, in the present study, the urine samples of T2D patients were considered to assess various metal levels in their samples relative to the non-diabetic (ND) apparently healthy control subjects. Mutual interactions of toxic and essential metals were also calculated.

SUBJECTS AND METHODS

Study subjects

The study population comprised 98 Pakistani Punjabi adults including 49 ND control subjects and 49 well-characterised T2D patients. The ND and T2D age (mean ± SD) was 45.2 ± 9.2 and 53.5 ± 10.4 years, respectively, with an age range of 35–80 years. A well-defined questionnaire was used to obtain information regarding occupation, physical activity, lifestyle patterns and dietary habits, past and current illness, and medication status. Anthropometric measurements of body height, weight and waist and hip circumference were also measured. T2D patients with nephropathy and malignancy were not included in the present study. After measuring height and weight, body mass index (BMI) (kg/m^2) was calculated by dividing weight (kg) by height in metre square (m^2). An informed consent was obtained from all subjects for their participation in this study. For the collection of urine and blood samples, camps were organised in Faisalabad, Pakistan. All subjects were informed to attend sample collection sites on a specific day in the morning after 12–14 hours overnight fast. During the fasting period, the subjects were only allowed to drink plain water. The study received approval from the institutional ethics committee.

Biological samples (blood and urine)

A venous blood sample was taken in a gel-containing vacutainer without an anticoagulant between 8 and 10 AM, after an overnight fast of 12–14 hours duration. The blood taken in vacutainers was allowed to clot and subsequently centrifuged for 10 minutes at 900 rpm to separate the serum. This was stored at −20ºC prior to downstream analysis. Serum were analysed for biochemical tests such as fasting glucose, cholesterol, triglycerides, total protein, *etc*. These biochemical parameters were estimated by colorimetric/spectrophotometric methods using commercial kits (DiaSys, Germany) on a semi-automatic clinical chemistry analyser (Microlab-300, Merck, Germany).

The urine samples of the same ND control subjects and T2D patients were also collected for the analysis of various metals. Different aliquots were stored at −20ºC until carrying out further analyses.

Urinary metals analysis

Varian double beam flame atomic absorption spectrophotometer (FAAS) (AA240 FS, Australia), with manual background correction, was employed for the determination of transition metals such as Zn, Cr, Cu, Mn, Pb, Ni and Fe and macronutrients such as Mg in the urine samples of T2D and control subjects. The analyses were carried out by using a hollow cathode lamp of respective metal under standard instrumental operational conditions. Acetylene gas was used as fuel.

Analytical procedure

For mineral estimation, 5 mL of each urine sample were digested in aqua regia and heated up to dryness. The residues were dissolved in 10 mL of concentrated HNO_3 and heated carefully until the solution turned colourless. It was then transferred into 25 mL flasks, and volumes were made up to the marks by de-ionised water. These sample solutions along with standards were aspirated to FAAS. For Mg estimation, serum samples were diluted (1 mL of urine in 25 mL of aqueous solution) with distilled water prior to their aspiration into an atomic spectrophotometer for analysis. The amount of each element in the sample was determined from its respective calibration curve. The analysis of each sample was made in duplicate, and the averaged value is reported in results.

RESULTS

General physical and biochemical characteristics of study subjects

General characteristics and laboratory findings of the studied human subjects are given in Table 1. A total of 49 ND control (males = 28 and females =21) subjects and 49 T2D (males = 20 and females = 28) patients were considered for selected metals analysis such as Cu, Mg, Cr, Fe, Zn, Ni and Mn using an atomic absorption spectrophotometer. The mean age of the ND control and T2D subjects was 45.1 ± 9.2 and 53.5 ± 10.4 years, respectively. Mean BMI (28 ± 0.7 kg/m^2) of T2D patients was significantly higher (p = 0.005) than the BMI (25.5 ± 0.34 kg/m^2) of the ND control group. There was also a significant difference (p = 0.004) in the mean value of waist hip ratio (WHR) (0.97 ± 0.08) of T2D patients when compared with the WHR (0.92 ± 0.6) of the ND control group. Fasting serum glucose and triglycerides were significantly ($p < 0.05$) higher in diabetics relative to their ND control subjects. On the other hand, fasting serum cholesterol, total proteins and creatinine were not significantly ($p < 0.05$) different between T2D and ND subjects (Table 1).

Urinary metal levels in T2D patients versus non-diabetic (ND) control subjects

In urinary metal analysis, the levels of Mg and Zn were found to be significantly higher ($p < 0.05$) in urine

Table 1: Anthropometric and biochemical characteristics of ND and T2D subjects

Subjects Parameters	Non-diabetic controls (N = 49)	T2D patients (N = 48)	*p*-value
BMI (kg/m^2)	25.5 ± 0.34	28 ± 0.7	0.005
WHR	0.92 ± 0.06	0.97 ± 0.08	0.004
Glucose (mg/dL)	88.7 ± 1.7	199.4 ± 10.7	< 0.0001
Cholesterol (mg/dL)	183.5 ± 6.6	176.3 ± 6.2	NS
Triglycerides (mg/dL)	127.9 ± 9.9	179.3 ± 15.07	0.005
Total proteins (g/dL)	7.7 ± 0.5	7.6 ± 0.13	NS
Creatinine (mg/dL)	0.73 ± 0.05	0.78 ± 0.04	NS

T2D: type 2 diabetes; ND = non-diabetic; BMI = body mass index; WHR = waist hip ratio; NS = non-significant.

samples of T2D compared to the ND control group. The mean values of Mg and Zn were 17.7 ± 1.6 and 0.46 ± 0.03 ppm for T2D patients, while in the case of the ND control group, the levels of Mg and Zn were obtained as 11.48 ± 1.3 and 0.33 ± 0.03 ppm, respectively. The statistical analyses show that the levels of these two metals, Mg and Zn, were 35 and 28% higher in the urine samples of T2D patients relative to their respective ND control subjects. On the other hand, in urine samples, the Fe level (1.16 ppm) in the T2D group was found to be lower ($p = 0.005$) than the value (1.77 ± 0.2 ppm) obtained for Fe in the urine samples of ND control human subjects. The Pb metal was found to be significantly higher ($p = 0.03$) in the urine of diabetics relative to the ND control group. The comparative levels of Pb in T2D patients and ND control subjects were 0.047 ± 0.003 and 0.036 ± 0.003 ppm, respectively. The calculated percent fold increase of Pb in the urine samples of T2D patients was about 29% as compared with the ND control subjects. The comparative urinary levels of Cu, Cr, Mn and Ni were higher in T2D patients than in ND control subjects, but these differences were not statistically significant (Fig. 1).

Fig. 2 shows a significant positive correlation of Pb with Zn ($r = 0.3284$, $p = 0.02$) and Mn ($r = 0.3648$, $p = 0.01$) in the urine samples of T2D patients. On the other hand, no correlation was found between Pb and other studied metals such as Mg, Fe, Cu, Cr, Mn and Ni. Similarly no relationship was found among Mg, Mn, Zn, Fe, Cu, Cr, Mn and Ni in the same diabetic urine samples.

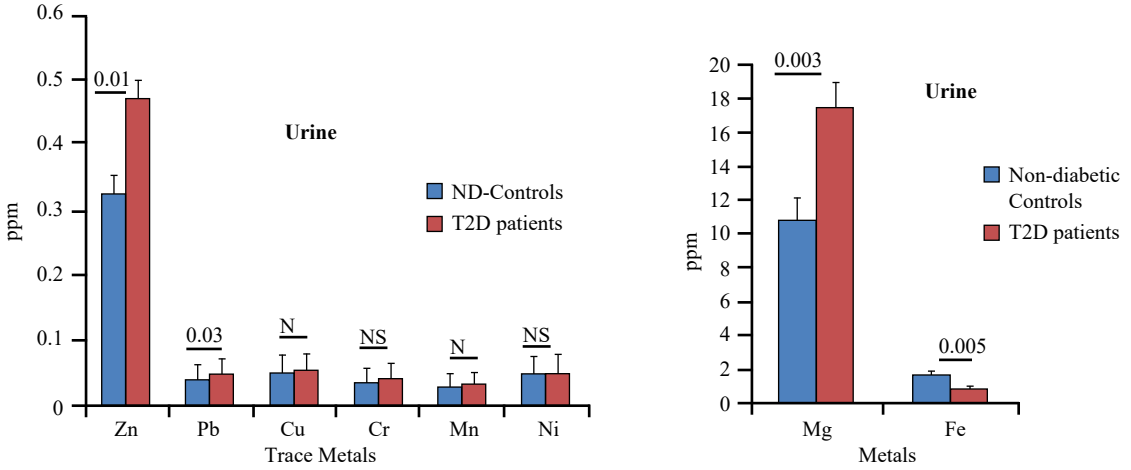

Fig. 1: Comparative urinary metals status of type 2 diabetics and ND control subjects. Zn = zinc; Pb = lead; Cu = copper; Cr = chromium; Mn = manganese; Ni = nickel; Mg = magnesium; Fe = iron; NS = not significant; T2D = type 2 diabetes; ND = non-diabetic.

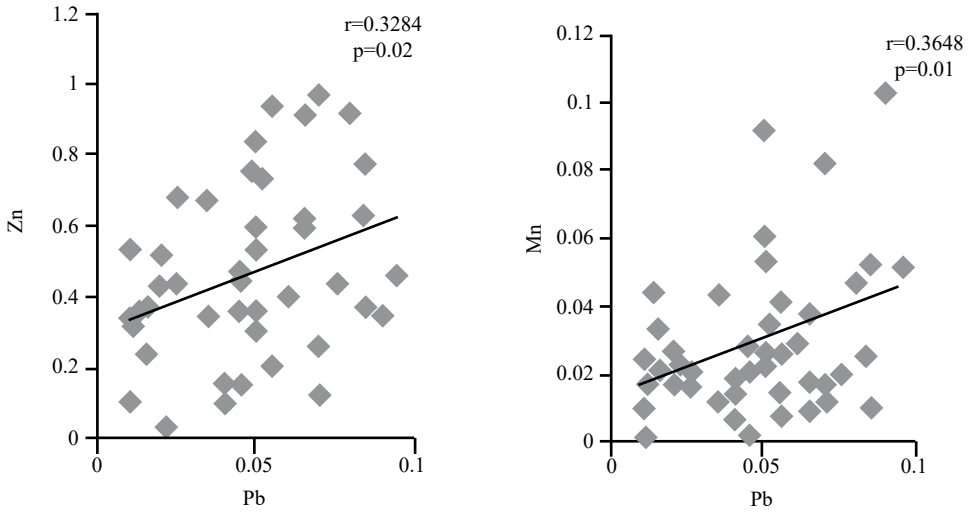

Fig. 2: Correlation analysis of Pb with Zn and Mn in the urine samples of T2D patients. Pb = lead; Zn = zinc; Mn = manganese; T2D = type 2 diabetes.

Non-diabetic control subjects and T2D patients were subdivided into ND control males versus ND control females and T2D males versus T2D females for further analysis to investigate gender-related changes in the metal concentrations.

Urinary metal levels of ND control male versus ND female subjects

The statistical analysis of the respective metal levels showed no significant difference in any of the above-mentioned metals between ND control male versus ND control female sub-groups. However, a close look shows that all metals including Zn, Cu, Mg, Pb, Cr, Mn and Ni were found to be higher in the urine samples of ND males, except Fe metals, which was 10-fold higher in the urines of ND females.

Urinary metal levels between T2D male and female patients

Similarly, no significant difference was found between T2D male and female patients with respect to urinary Mg, Pb metals and other trace elements (TEs) including Cu, Zn, Fe, Cr, Mn and Ni. In this case, the urinary levels of Cu, Fe, Cr and Ni were observed non-significantly higher in T2D male versus T2D female patients. On the other hand, Zn, Mg and Mn concentrations were marginally higher in T2D females while comparing with T2D male patients.

DISCUSSION

It has already been established that hyperglycaemia in diabetic patients is linearly linked with the production of advanced glycation end products of several proteins and highly ROS such as H_2O_2, OH and O_2- (18). Metals are central to hold the bioactivity of numerous enzymes and proteins on which life is based. Derangements in the metal (*eg*, Zn, Mg, Cu, Mn, Cr, *etc*) metabolism could lead to the pathogenesis of diabetes. Imbalances in the status of essential metals have been reported as a worsening incidence in the progression of diabetes (19). Several previously published studies manifested the functions of transition and TEs such as Fe, Zn, Mg, Cu, Mn and Cr in insulin action and carbohydrate metabolism. The alterations in these elements attributed to hyperglycaemia and enhanced magnitude of protein glycation in diabetic state. A case-control study revealed alterations in the metabolism of metallic elements such as Zn, Mg, Cu, Mn and Cr in diabetic patients relative to their ND control subjects (20).

In the present study, an increased urinary level of Mg was observed in T2D patients compared with the ND control subjects. These findings are supported by a previously published report (21), in which negative correlation was reported between serum Mg and urinary Mg levels in diabetic patients. This correlation revealed a decrease of serum Mg with concomitant increase of the urinary Mg level. Renal Mg excretion may be regulated by insulin. The mechanism of the urinary loss of Mg may result probably from the reduced capacity of tubular reabsorption of Mg due to osmotic action of glycosuria (9). Previous studies have also revealed an association between hypomagnesaemia and elevated glucose levels. Yet, another study correlated Mg deficiency with fat accumulation in various body tissues, which subsequently leads to obesity-related metabolic disorders (22). This provides a further explanation to high presence (loss) of Mg in the urine of T2D patients as more than 60% of them are obese.

Urinary Fe concentration was less in T2D patients than ND control subjects. This agrees with the previously published results (20), in which concurrent elevation of the serum iron level with decrease urinary excretion of Fe was observed in diabetic patients. Several studies manifested possible association between high iron stores and various metabolic parameters such as blood serum insulin, glucose, dyslipidaemia and obesity (23). Iron is a potential catalyst that produces ROS, which subsequently develop oxidative stress and damages healthy tissues, thereby increasing the risk of T2D (24).

Copper concentrations in various biofluids including urine have been found to be altered in diabetic subjects. The higher urinary level of Cu was observed in T2D patients compared with the ND control subjects in the present study, though the difference was not significant. These results are consistent with the previously published results (25). It has also been manifested that Cu deficiency is an important cause of adiposity development (26).

The urine Zn level was significantly higher in T2D patients compared with the ND control subjects. Previous clinical studies have reported excessive loss of Zn through urine with decreased values in the blood serum in diabetes as compared with ND control subjects (20, 27). The decreased gastrointestinal absorption with increased urinary excretion of Zn in diabetes may lead to hypozincaemia. It has been suggested that prolonged hyperglycaemia may adversely affect the transport of Zn back into the renal tubules. In addition to this, mutation in the Znt8 transporter gene (*SLC30A8*) contributes to

Zn deficiency. Zn deficiency in diabetes may be associated with increased oxidative stress and tissue damaging (20, 28). Furthermore, Zn is required for the synthesis of insulin hexamer, which is vital to keep the blood sugar level in normal range. It has been reported that mutation in *SLC30A8* gene is associated with T2D (8, 29).

In the present study, the level of Cr in the urine samples of T2D patients was higher than those of respective ND control subjects, but the difference was not significant. It has been extensively studied that Cr takes part in increasing receptors number and binding of insulin to insulin receptors in target organ (30). Trivalent chromium (Cr III) is effective for glucose uptake by the cells. Chromium urinary excretion is expedited in hyperglycaemic condition (30, 31). Anderson *et al*. reported antioxidant effect of the combined supplementation of Cr and Zn in T2D patients (28). Manganese urinary level was higher than their respective ND healthy control subjects. There is no data available in literature which shows comparative urinary excretion of Mn in T2D patients relative to their ND control group. Nevertheless, studies report the possible involvement of Mn deficiency in the pathogenesis of DM (20). However, it has been reported that oral Mn administration improves the insulin sensitivity in diabetic patients (32).

In this study, the mean levels of two toxic metals such as Pb and Ni were studied in T2D patients versus ND control subjects. The results of the present study revealed that the urinary level of Pb was significantly higher in T2D patients as compared with the ND control subjects. Lead is a heavy transition metal that is harmful to most of the human tissues and interferes with the body's physiological functions (33, 34). Lead is ubiquitous environmental toxin that is associated with a wide range of biochemical and physiological dysfunctions (26). A recent study revealed that environmental exposure to Pb is contributing in the development of neuropathy-related complications in T2D patients (35). Several studies have manifested a positive correlation between environmental Pb exposure and reduction in renal function efficiency, and Pb chelating therapies may be effective to improve renal function in ND subjects with chronic kidney disorders (36). Lead also contributes to various metabolic disorders including obesity by substituting vital TEs, *eg*, Zn, Cr and Cu with sequential depletion of anti-oxidative potential of blood in individuals who were frequently exposed to toxic heavy metals including Pb (26).

In the present study, Pb shows positive associations with two essential TEs such as Zn and Mn in the urine specimens of T2D patients, as shown in Fig. 2. Both these TEs (Zn and Mn) act as cofactors for the activity of several enzymes. The increased interactive urinary excretion of TEs such as Zn with toxic Pb metal might adversely affect the levels of these two essential metals in blood sera of T2D patients. As interactions of toxic metals including Pb with essential trace elements have been extensively studied (26). Continuous loss of Zn and Mn over time through urination causes deficiency in TEs levels that may demonstrates the decrease of Zn and Mn in blood of diabetic patients (37).

The urinary Ni level was not different between T2D patients and ND control subjects. There is scant information available in literature; some human and animal studies indicate that Ni deprivation lowers the blood plasma glucose level, which subsequently alters metallic elements (Zn, Fe and Cu) tissue distribution and metabolism (38). An animal study shows that Ni induces oxidative stress in body organs particularly in kidney (39).

In the present study, no association was found between the urinary levels of aforementioned metallic elements with age, gender, clinical and anthropometric characteristics. Kazi *et al*. reported sex- and age-related impact on the bioavailability of TEs (20). The reason for the disparity in the findings is not yet clear and may be attributed to sample size. Simultaneously, the analysis of metals in various biological specimens including urine has been conducted in several previous studies. Among them, blood plasma and urine may be more beneficial, since derangements of metals in blood seems to be reflected in urine of diabetic patients (10). Moreover, urine can be obtained easily and non-invasively compared to with other biofluids such as blood plasma/serum, lacriminal fluid and synovial fluid (8); therefore, urine was used in this study.

CONCLUSION

Based on the findings obtained in this study, it could be concluded that the normal levels of essential metals are disturbed in T2D patients, which are reflected in their urine samples. The status of essential TEs in various body tissues is influenced by renal excretion. Alteration in the level of one metal may influence the normal levels of other metals. As in the present study, statistical analysis demonstrated a linear relationship between a toxic metal Pb with two essential TEs, *ie*, Zn and Mn in the urine samples of T2D patients. It can also be suggested that toxic metals such as Pb may have a role to induce renal tubular dysfunction in the study diabetic

population. Subsequently, dysfunctional kidneys may become a potential source for the loss of several essential TEs through urine voiding rather than their retention in blood plasma/serum in order to retain the homeostasis of blood and other tissues. Consistent with previous reports, in the present study the high level of Zn was observed in the urine samples of T2D patients. This high urinary excretion of Zn results in decrease levels of Zn in blood plasma/serum, which is the sign of disruptions in the Zn-based antioxidant mechanism. However, the levels of essential trace elements vary in samples studied among individuals. Thus, it may be worth exploring further interactions among various types of metals in T2D patients using large number of urine samples.

Briefly in the present study, in addition to alterations in metal status in the urine samples of T2D patients, statistical analysis demonstrates relationships between more than two metallic elements, which after further large population-based prospective studies may prove to have scientific as well as practical implications in understanding and controlling diabetes or its complications.

AUTHORS' NOTE

The authors declare that they have no conflict of interest about this research publication. ARK conducted and wrote the initial drafts and FRA supervised and critically revised/approved the manuscript.

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REFERENCES

1. Sundsten T, Ortsäter H. Proteomics in diabetes research. Mol Cell Endocrinol 2009; **297:** 93–103.
2. Moller DE. New drug targets for type 2 diabetes and the metabolic syndrome. Nature 2001; **414:** 821–7.
3. Khan AR, Awan FR. Review: mining of protein based biomarkers for type 2 diabetes mellitus. Pak J Pharma Sci 2012; **25:** 889–901.
4. Leahy JL. Pathogenesis of type 2 diabetes mellitus. Arch Med Res 2005; **36:** 197–209.
5. Hu FB, Manson JE, Stampfer MJ, Colditz G, Liu S, Solomon CG et al. Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. New Eng J Med 2001; **345:** 790–7.
6. Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. New Eng J Med 2001; **344:** 1343–50.
7. Kodama S, Saito K, Tanaka S, Maki M, Yachi Y, Sato M et al. Influence of fat and carbohydrate proportions on the metabolic profile in patients with type 2 diabetes: a meta-analysis. Diab Care 2009; **32:** 959–65.
8. Khan AR, Awan FR. Metals in the pathogenesis of type 2 diabetes. J Diab Metab Disord 2014; **13:** 16.
9. Swaminathan R. Magnesium metabolism and its disorders. The Clin Biochem Rev 2003; **24:** 47.
10. Afridi HI, Kazi TG, Kazi N, Jamali MK, Arain MB, Jalbani N et al. Potassium, calcium, magnesium, and sodium levels in biological samples of hypertensive and nonhypertensive diabetes mellitus patients. Biol Trac Elem Res. 2008; **124:** 206–24.
11. Brender JR, Hartman K, Nanga RPR, Popovych N, de la Salud Bea R, Vivekanandan S et al. Role of zinc in human islet amyloid polypeptide aggregation. J Am Chem Soc. 2010; **132:** 8973–3.
12. Forte G, Bocca B, Peruzzu A, Tolu F, Asara Y, Farace C et al. Blood metals concentration in type 1 and type 2 diabetics. Biol Trac Elem Res 2013; **156:** 79–90.
13. Król E, Krejpcio Z. Chromium (III) propionate complex supplementation improves carbohydrate metabolism in insulin-resistance rat model. F Chem Toxicol 2010; **48:** 2791–6.
14. Raj S, Rajan G. Correlation between elevated serum ferritin and HbA1c in type 2 diabetes mellitus. Int J Res Med Sci 2013; **1:** 12.
15. Kundu D, Roy A, Mandal T, Bandyopadhyay U, Ghosh E, Ray D. Relation of iron stores to oxidative stress in type 2 diabetes. Nig J Clin Prac 2013; **16:** 100–3.
16. Akinloye O, Ogunleye K, Oguntibeju O. Cadmium, lead, arsenic and selenium levels in patients with type 2 diabetes mellitus. Afr J Biotech 2010; **9:** 5189–95.
17. Navas-Acien A, Silbergeld EK, Pastor-Barriuso R, Guallar E. Arsenic exposure and prevalence of type 2 diabetes in US adults. JAMA 2008; **300:** 814–22.
18. Nishikawa T, Araki E. Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. Anti Red Signal 2007; **9:** 343–53.
19. Viktorínová A, Tošerová E, Križko M, Ďuračková Z. Altered metabolism of copper, zinc, and magnesium is associated with increased levels of glycated hemoglobin in patients with diabetes mellitus. Metab. 2009; **58:** 1477–82.
20. Kazi TG, Afridi HI, Kazi N, Jamali MK, Arain MB, Jalbani N et al. Copper, chromium, manganese, iron, nickel, and zinc levels in biological samples of diabetes mellitus patients. Biol Trac Elem Res 2008; **122:** 1–18.
21. Chinyere NA, Opara UCA, Henrieta E, Nathaniel U. Serum and urine levels of chromium and magnesium in type 2 diabetics in Calabar, Nigeria. Mal J Nutr 2005; **11:** 133–142.
22. Song CH, Song IK, Ju SY, Ock SM. Serum magnesium level is negatively associated with fasting serum glucose level in Korean adults. Biol Trac Elem Res 2011; **143:** 612–8.
23. Rajpathak S, Ma J, Manson J, Willett WC, Hu FB. Iron intake and the risk of type 2 diabetes in women: a prospective cohort study. Diab Care 2006; **29:** 1370–6.
24. Fernández-Real JM, López-Bermejo A, Ricart W. Cross-talk between iron metabolism and diabetes. Diabetes 2002; **51:** 2348–54.
25. Ito S, Fujita H, Narita T, Yaginuma T, Kawarada Y, Kawagoe M et al. Urinary copper excretion in type 2 diabetic patients with nephropathy. Nephron. 2001; **88:** 307–12.
26. Padilla MA, Elobeid M, Ruden DM, Allison DB. An examination of the association of selected toxic metals with total and central obesity indices: NHANES 99-02. Int J Environ Res Pub Health. 2010; **7:** 3332–47.
27. Nasli-Esfahani E, Faridbod F, Larijani B, Ganjali MR, Norouzi P. Trace element analysis of hair, nail, serum and urine of diabetes mellitus patients by inductively coupled plasma atomic emission spectroscopy. Iran J Diab Lipid Disord 2011; **10:** 1–9.
28. Anderson RA, Roussel AM, Zouari N, Mahjoub S, Matheau JM, Kerkeni A. Potential antioxidant effects of zinc and chromium supplementation in people with type 2 diabetes mellitus. J Am Coll Nutr 2001; **20:** 212–8.
29. Xiang J, Li XY, Xu M, Hong J, Huang Y, Tan JR et al. Zinc transporter-8 gene (SLC30A8) is associated with type 2 diabetes in Chinese. J Clin Endocrinol Metab 2008; **93:** 4107–2.
30. Cefalu WT, Hu FB. Role of chromium in human health and in diabetes. Diab Care 2004; **27:** 2741–51.
31. Diwan A, Pradhan A, Lingojwar D, Krishna K, Singh P, Almelkar S. Serum zinc, chromium and magnesium levels in type 2 diabetes. Int J Diab Develop Ctries. 2006; **26:** 122–3.

32. Ekmekcioglu C, Prohaska C, Pomazal K, Steffan I, Schernthaner G, Marktl W. Concentrations of seven trace elements in different hematological matrices in patients with type 2 diabetes as compared to healthy controls. Biol Trac Elem Res 2001; **79:** 205–19.
33. Kazi TG, Jalbani N, Kazi N, Arain MB, Jamali MK, Afridi HI et al. Estimation of toxic metals in scalp hair samples of chronic kidney patients. Biol Trac Elem Res. 2009; **127:** 16–27.
34. Kazi TG, Jalbani N, Kazi N, Jamali MK, Arain MB, Afridi HI et al. Evaluation of toxic metals in blood and urine samples of chronic renal failure patients, before and after dialysis. Renal Fail 2008; **30:** 737–45.
35. Lin J, Lin-Tan D, Yu C, Li Y, Huang Y, Li K. Environmental exposure to lead and progressive diabetic nephropathy in patients with type II diabetes. Kid Int 2006; **69:** 2049–56.
36. Yu CC, Lin JL, Lin-Tan DT. Environmental exposure to lead and progression of chronic renal diseases: a four-year prospective longitudinal study. J Am Soc Nephrol 2004; **15:** 1016–22.
37. Bamgbose O, Opeolu B, Bamgbose J. Levels of cadmium, lead and zinc in urine of randomly selected smokers and non-smokers residents of Abeokuta City, Nigeria. Research J Appl Sci 2007; **2:** 192–7.
38. Serdar MA, Bakir F, Haşimi A, Çelik T, Akin O, Kenar L et al. Trace and toxic element patterns in nonsmoker patients with noninsulin-dependent diabetes mellitus, impaired glucose tolerance, and fasting glucose. Int J Diab Develop Ctries. 2009; **29:** 35.
39. Kubrak OI, Husak VV, Rovenko BM, Poigner H, Mazepa MA, Kriews M et al. Tissue specificity in nickel uptake and induction of oxidative stress in kidney and spleen of goldfish, exposed to waterborne nickel. Aqua Toxicol 2012; **118:** 88–96.

ORIGINAL ARTICLE

Personal and Behavioural Characteristics of Seat Belt Non-users in Slovenia

M Bilban[1,2]

ABSTRACT

Objective: *In turn, personal and behavioural characteristics found in drivers who use seat belts affect general traffic safety. The objective of our study was to determine the risk factors that affect seat belt use across the observed behaviour-related groups of adult citizens of Slovenia.*
Methods: *Data were collected in late spring 2001, 2004, 2008 and 2012 in a cross-sectional survey, which is conceptually part of a wider international project in the frame of the Countrywide Integrated Non-communicable Diseases Intervention Programme. A stratified random sample was drawn from the Central Population Registry of the Republic of Slovenia.*
Results: *The most significant results of our study showed that the important risk factors for seat belt use in all three periods of examination are as follows: gender, age and education level. We have found seat belt use in the front and the back car seats among adult citizens of Slovenia to be gradually becoming more and more prevalent. However, there are still a worrisome percentage of people who never use seat belts.*
Conclusion: *We will thus have to continue implementing existing activities intended to raise awareness and seat belt use. In particular, planning stages for various public-health measures should see us focussing on at-risk population groups. Further data will have to be obtained in order for us to be able to exactly evaluate behaviour-affecting risk factors (eg, psycho-physical wellbeing, use of medicinal products).*

Keywords: Behaviour-related groups, risk factors, seat belt, traffic safety.

INTRODUCTION

The seat belt is a vehicle safety device intended to protect the driver and passengers from serious injuries that could result from a crash or sudden deceleration of the vehicle. Failure to use the seat belt in an automobile is a risk factor for grave injuries in traffic accidents (1). According to the results of extensive studies, use of a seat belt decreases the probability of mortal injury in a traffic accident by over 40%, regardless of the speed or the driver's age (2).

Use of a seat belt is affected by a number of factors connected to personal and behavioural characteristics (3–5). A previous research finding (3–5) shows that young drivers are less likely to use the seat belt than older drivers and men are less likely to use the seat belt than women.

Based on European legislation, a recommendation by the European Commission sets a number of requirements regarding seat belts in vehicles and their mandatory use during driving. At the national level, the Road Traffic Rules Act (6) prescribes seat belts in a motor vehicle must be used by passengers in all seats that have them and extends the authority to monitor and punish the failure to use a seat belt to city wardens. The only persons exempt from having to use a seat belt are those who are able to demonstrate, with a valid medical certificate, that medical reasons prevent them from using a seat belt (6).

Numerous studies in Slovenia and abroad show that appropriate legislation is one of the key factors of seat belt use—in all countries where such legislation was modernised, the use of seat belts has increased both in car drivers and passengers (7–10).

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Our study's objective was to define risk factors that affect seat belt use for each of the three behavioural categories in adult citizens of Slovenia. A side objective was to use the data gathered (in 2001, 2004, 2008 and 2012) to evaluate the effectiveness of public-health measures associated with seat belt use.

SUBJECTS AND METHODS

Data collection

Data were collected in late spring 2001, 2004, 2008 and 2012 in a cross-sectional survey, which was conceptually a part of a wider international project in the frame of the Countrywide Integrated Non-communicable Diseases Intervention (CINDI) Programme. A self-administered postal questionnaire was used based on the CINDI Health Monitor (CHM) Core Questionnaire. The research protocol for the survey was approved by the Ethical Committee of the Republic of Slovenia.

Observed outcomes

The patterns of seat belt use were assessed on the basis of two questions: (a) 'Do you use a seat belt when driving or as a passenger in the front seat?' (1—never; 2—sometimes; 3—almost always) and (b) 'Do you use a seat belt in the back seat?' (1—never; 2—sometimes; 3—almost always; 4—there is no seat belt in the back seat and 5—I never travel in the back of the car). On the basis of cross-classification of both questions, participants were classified in three groups according to the level of hazardous behaviour regarding seat belt use (observed outcome): 1—low-risk group (almost always in the front seat, and almost always in the back seat, or they never travel in the back of the car); 2—moderate-risk group (almost always in the front seat, and never, or sometimes in the back seat, or there is no seat belt in the back seat) and 3—high-risk group (never, or only sometimes in the front seat, regardless the answers regarding using seat belt in the back seat).

Data analysis

For the analysis of non-use of seat belts in the front seat, the total sample was used. Participants who reported that they never travel in the back of the car, or that there was no seat belt in the rear seat of their car, were excluded from the analysis of non-use of seat belts in the rear seat.

The observed outcome was related to (risk factors) gender; age: 25–29, 30–39, 40–49, 50–59 or 60–64 years; level of education: uncompleted primary (less than 8 years of education), primary (8 years), vocational (10–11 years), secondary (12 years), college (14–15 years) or university (16 years or more of education); employment: employed, not employed (housekeeper/student, pensioner/disability pensioner, involuntary unemployed— job seeker); social class (self-classification): lower, labour, middle, upper-middle or upper; and geographical region: western, central or eastern for each observed year.

Estimates of prevalence for three levels of observed outcome were assessed for each population subgroup regarding above-mentioned characteristics, whereas the strength of the association between the occurrence of observed outcome and each of selected risk factors was univariate estimated using the Chi-squared test.

Multivariate logistic regression analysis was used to estimate the strength of the association between the occurrences of moderate-risk in comparison to low-risk, and high-risk in comparison to low-risk behaviour and selected risk factors. In all statistical tests p-value 0.05 or less was considered significant. SPSS statistical package for Windows Version 21.0 (SPSS Inc., Chicago, IL, USA) was used for analysis.

RESULTS

Description of data

The questionnaires of 8861 (CHM, 2001), 8232 (CHM, 2004), 5885 (CHM, 2008) and 7562 (CHM, 2012) respondents were eligible for analysis (Table 1).

Results of cross-classification of participants according to their answers on questions about fastening with seat belt in the front and in the back seat by year are presented in (Table 1).

Statistical analysis

According to the different levels of hazardous behaviour: non-hazardous (low-risk) behaviour was observed in 46.4% (CHM, 2001), 50.9% (CHM, 2004), 66.8% (CHM, 2008) and 69.7% (CHM, 2012) participants; hazardous (moderate-risk) behaviour was observed in 46.2% (CHM, 2001), 41.1% (CHM, 2004), 28.3% (CHM, 2008) and 26.3% (CHM, 2012) participants; and very hazardous (high-risk) behaviour was observed in 5.5% (CHM, 2001), 4.5% (CHM, 2004), 1.9% (CHM, 2008) and 1.5% (CHM, 2012) participants.

Estimates of prevalence (%) for three levels of hazardous behaviour related to seat belt use in a car, in different population subgroups in participants of the survey by observed year, are presented in Table 2.

Table 1: Cross-classification of participants according to their answers on questions about seat belt use in the front and in the back seat by observed years

Front seat	Back seat					
	Never	Sometimes	Almost always	No seat-belt in the back seat	Never travel in the back of the car	Total
2001 (n = 8861)						
Never	46	5	0	1	8	60
Sometimes	283	58	0	22	74	437
Almost always	1873	1746	2130	555	2060	8364
Total	2202	1809	2130	578	2142	8861
2004 (n = 8232)						
Never	33	5	0	0	3	41
Sometimes	228	57	0	6	56	347
Almost always	1427	1854	2673	224	1666	7844
Total	1688	1916	2673	230	1725	8232
2008 (n = 5885)						
Never	21	1	0	0	0	22
Sometimes	55	25	0	2	10	92
Almost always	622	1033	3288	61	767	5771
Total	698	1059	3288	63	777	5885
2012 (n = 7562)						
Never	17	2	0	0	1	20
Sometimes	60	28	0	0	11	99
Almost always	564	1438	4542	35	864	7443
Total	**641**	**1468**	**4547**	**35**	**876**	**7562**

Results of multivariate logistic regression analysis between moderate-risk behaviours related to seat belt use in a car in comparison to low-risk behaviour by observed year are presented in (Table. 3).

Results of multivariate logistic regression analysis between high-risk behaviours related to seat belt use in a car in comparison to low-risk behaviour by observed year are presented in (Table. 4).

DISCUSSION

The most significant results of our study have shown that the major risk factors affecting seat belt use in all three periods under investigation were: gender, age and education level.

In the period under investigation, results have shown an increase in the number of drivers who almost always use the seat belt on either of the front seats. The trend is particularly significant in seat belt use on back seats (from 2001 to 2008, the share increased by 35.0%). At the same time, the percentage of drivers who never use a seat belt, neither on the front nor on the back seats, is decreasing.

It is a fact that seat belt use prevents traffic accident injuries and/or reduces their severity. Numerous studies show an increased awareness regarding the use and perception of the use of seat belts in professional drivers. Regarding traffic accidents, the European Commission notes that numerous fatalities and cases of serious injury in traffic accidents could be averted if everybody used a seat belt while travelling by car, resulting in a great decrease of the costs associated with such accidents. A number of studies have found that a 100% seat belt use rate would halve the number of traffic accident fatalities while reducing the number of serious injuries by as much as 70%. At high speeds, the seat belt can only cause surface injuries to the body and skin, minor bone fractures; only in exceptional cases does seat belt use result in serious internal injuries. Injuries potentially suffered with seat belt use are simply incomparable to those potentially suffered by drivers who fail to use the seat belt (11).

In case of a traffic accident, failure to use a seat belt may cause the driver to crash into the steering wheel, the windshield or other parts of the vehicle's interior, or

Table 2: Estimates of prevalence (%) for three levels of hazardous behaviour related to seat belt use in a car, in different population subgroups in participants of the survey by years, Slovenia

Population groups	Low risk level Year (%)				*p*	Moderate risk level Year (%)				*p*	High risk level Year (%)				*p*
	2001	2004	2008	2012		2001	2004	2008	2012		2001	2004	2008	2012	
Gender															
Men	25.8	24.2	20.9	29.1	< 0.001	34.9	31.0	14.6	19.6	< 0.001	41.5	35.3	11.9	11.3	0.010
Women	21.2	24.0	23.9	30.8		37.7	30.4	15.3	16.6		50.1	33.5	7.0	9.4	
Age (years)															
25–29	25.6	24.9	22.1	27.4	< 0.001	36.3	33.1	16.3	14.3	< 0.001	41.9	42.4	7.8	7.8	0.001
30–39	26.3	24.5	21.8	27.4		37.5	30.4	14.1	18.1		46.4	33.9	9.4	10.3	
40–49	23.8	24.3	21.9	30.1		40.9	29.6	13.4	16.1		49.8	32.8	9.8	7.5	
50–59	21.5	23.5	24.1	30.9		32.0	30.4	17.6	20.0		36.1	33.2	14.9	15.9	
60–64	20.0	24.0	22.1	33.9		32.7	31.1	13.3	22.9		44.8	25.9	8.6	20.7	
Education level															
Uncompleted primary	34.6	30.8	19.8	14.8	< 0.001	54.6	28.8	9.2	7.5	< 0.001	53.3	35.0	6.7	5.0	0.104
Primary	26.6	26.1	22.3	25.0		46.5	28.2	13.8	11.5		48.9	26.7	11.5	13.0	
Vocational	27.5	26.2	22.5	23.8		42.3	31.4	14.3	12.0		47.4	34.2	9.7	8.7	
Secondary	19.6	21.5	22.2	36.8		31.0	31.3	15.6	22.2		37.4	36.8	11.2	14.6	
college	23.0	23.8	23.0	30.2		37.4	29.7	13.7	19.2		48.3	38.2	5.6	7.9	
University	18.2	19.4	24.0	38.4		27.1	29.9	17.6	25.4		45.3	34.0	11.3	9.4	
Employment															
No	22.9	23.9	20.4	32.8	< 0.001	37.3	28.8	13.1	20.8	< 0.001	42.4	29.7	10.0	17.9	< 0.001
Yes	23.6	24.4	24.5	27.6		36.2	31.8	16.0	16.0		44.5	36.6	10.6	8.3	
Social class (self-classification)															
Lower	21.1	18.7	25.3	34.9	< 0.001	32.6	29.7	17.6	20.1	< 0.001	27.0	35.1	10.8	27.0	< 0.001
Labour	22.9	22.8	23.0	31.3		39.9	27.3	14.5	18.3		40.7	31.8	12.5	15.0	
Middle	23.7	23.8	22.2	30.4		35.3	31.2	15.1	18.3		46.5	35.9	8.5	9.1	
Upper-middle	23.7	27.2	22.7	26.4		32.2	35.5	15.3	16.9		44.0	40.5	11.2	4.3	
Upper	36.3	29.5	11.0	23.3		40.0	37.1	8.6	14.3		81.8	18.2	0.0	0.0	
Geographic region															
Western	24.1	24.3	21.7	29.9	0.332	35.0	29.6	14.8	20.6	< 0.001	43.8	34.0	10.4	11.8	0.609
Central	23.3	23.4	22.3	30.9		34.9	31.3	16.2	17.6		47.6	35.2	9.7	7.6	
Eastern	22.9	24.4	23.0	29.6		38.4	30.8	14.3	16.5		43.1	34.8	10.4	11.7	

Levels of hazardous behaviour: **low risk** (almost always in the front seat, and almost always in the back seat, or they never travel in the back of the car); **moderate risk** (almost always in the front seat and never or sometimes in the back seat, or there is no seat belt in the back seat) and **high risk** (never, or only sometimes in the front seat, and all answers regarding using a seat belt in the back seat); $p \leq 0.05$.

throw the driver out of the vehicle (11). Our research results have shown 0.8% (CHM, 2001) to 0.3% (CHM, 2012) of adult Slovenian citizens never to use the seat belt on the front seats. Failure to use the seat belt is even more frequent on back seats: from 24.5% (CHM, 2001) to 8.3% (CHM, 2012).

In the European Union (EU) alone, failure to use the seat belt claims almost 7000 lives each year. Traffic safety planners in Europe are thus convinced that failure to use seat belts should be punished even more severely (12). The stated reasons for failure to use seat belts are as follows: 34% of drivers forget to use it; 22% are only driving a short distance and 10% do not use it because it bothers them while they are driving (13). As indicated by the results of our study, efforts in Slovenia should be focussed on the groups that are most at risk, *ie*, those between 25 and 29 years of age, those with a low level of education and those living in western Slovenia. Considering the results noted in each year of the study, the measures already implemented have been successful. However, we still have to work to determine the population groups that are at the highest risk and design appropriate measures with them in mind.

In addition to warning lights, newer cars often feature an audible warning signal. Estimates in Sweden indicate that an effective system that warns the vehicle's occupants to use their seat belts may decrease the fatality rate in traffic accidents by about 20%. In the EU, that would mean a difference of approximately 4000 deaths every year (13).

Table 3: Results of logistic regression analysis of risk factors for moderate-risk behaviour related to seat belt use in a car in comparison to low-risk behaviour in participants of the survey Slovenia

Risk factor	Observed category	Reference category	Moderate-risk to low-risk level							
			Year 2001		Year 2004		Year 2008		Year 2012	
			OR	*p*	OR	*p*	OR	*p*	OR	*p*
Gender	Men	Women	1.55	< 0.001	1.22	< 0.001	1.08	0.245	0.94	0.326
Age (years)	25–29	50–59	1.17	< 0.001	1.13	< 0.001	1.20	< 0.001	1.11	< 0.001
	30–39	50–59	1.08	< 0.001	1.04	0.025	1.05	0.061	1.10	< 0.001
	40–49	50–59	1.07	< 0.001	0.99	0.527	0.98	0.246	0.98	0.226
	60–64	50–59	0.94	0.008	0.95	0.018	0.87	< 0.001	0.92	0.001
Education level	Uncompleted primary	University	0.99	0.768	0.94	0.008	1.00	0.876	1.02	0.553
	Primary	University	0.98	0.513	0.95	0.007	1.01	0.752	1.00	0.164
	Vocational	University	0.98	0.471	0.96	0.018	1.02	0.553	1.00	0.329
	Secondary	University	1.00	0.709	0.99	0.791	1.01	0.456	1.02	0.226
	College	University	1.02	0.258	0.98	0.318	1.01	0.915	1.02	0.214
Employment	Yes	No	1.02	0.657	0.91	0.003	0.97	0.442	1.02	0.514
Social class (self-classification)	Labour	Lower	1.16	0.312	0.82	0.242	0.84	0.336	1.10	0.564
	Middle	Lower	1.04	0.827	0.86	0.390	1.00	0.988	1.20	0.279
	Upper-middle	Lower	0.93	0.666	0.83	0.307	1.01	0.981	1.33	0.141
	Upper	Lower	0.84	0.506	0.90	0.709	1.35	0.523	1.55	0.229
Geographic region	Western	Eastern	1.03	0.102	1.08	<0.001	1.13	<0.001	1.12	<0.001
	Central	Eastern	1.01	0.442	1.06	0.001	1.10	<0.001	1.06	0.008

Abbreviations: OR = odds ratio; levels of hazardous behaviour—**low risk** (almost always in the front seat and almost always in the back seat or they never travel in the back of the car) and **moderate risk** (almost always in the front seat and never or sometimes in the back seat, or there is no seat belt in the back seat); $p \leq 0.05$.

Table 4: Results of logistic regression analysis of risk factors for moderate-risk/high-risk behaviour related to seat belt use in a car in comparison to low-risk behaviour in participants of the survey Slovenia

Risk factor	Observed category	Reference category	High-risk to low-risk level							
			Year 2001		Year 2004		Year 2008		Year 2012	
			OR	*p*	OR	*p*	OR	*p*	OR	*p*
Gender	Men	Women	0.54	< 0.001	0.40	< 0.001	0.22	< 0.001	0.37	< 0.001
Age (years)	25–29	50–59	1.24	< 0.001	1.38	< 0.001	1.12	0.195	1.27	0.003
	30–39	50–59	1.19	< 0.001	1.19	< 0.001	1.10	0.179	1.15	0.044
	40–49	50–59	1.13	0.002	1.11	0.018	1.06	0.439	1.00	0.821
	60–64	50–59	0.95	0.478	0.85	0.060	0.79	0.097	1.00	0.452
Education level	Uncompleted primary	University	1.17	0.002	1.25	< 0.001	1.23	0.063	1.22	0.098
	Primary	University	1.12	0.006	1.13	0.013	1.13	0.143	1.22	0.010
	Vocational	University	1.13	< 0.001	1.15	< 0.001	1.16	0.026	1.20	0.007
	Secondary	University	1.08	0.021	1.12	0.002	1.17	0.013	1.15	0.023
	College	University	1.08	0.061	1,13	0.004	1.04	0.695	1.13	0.147
Employment	Yes	No	0.90	0.177	0.89	0.157	0.92	0.612	1.03	0.792
Social class (self-classification)	Labour	Lower	1.13	0.738	0.54	0.060	1.00	1.000	0.495	0.067
	Middle	Lower	1.48	0.304	0.67	0.228	1.00	0.980	0.40	0.028
	Upper-middle	Lower	1.94	0.111	0.85	0.659	1.82	0.391	0.36	0.090
	Upper	Lower	3.41	0.022	0.53	0.425	–	–	–	0.998
Geographic region	Western	Eastern	1.09	0.039	1.08	0.089	1.14	0.097	1.11	0.195
	Central	Eastern	1.00	0.941	1.00	0.948	1.00	0.651	0.89	0.186

Abbreviations: OR = odds ratio; levels of hazardous behaviour—**low risk** (almost always in the front seat and almost always in the back seat, or they never travel in the back of the car) and **high risk** (never or only sometimes in the front seat and all answers regarding using seat-belt in the back seat); $p \leq 0.05$.

A driver and passenger in the front seat who are wearing their seat belts can usually walk from a 64 km/hour head-on collision with minor injuries. However, if the driver and passenger are not strapped in, the consequences of a collision at such speed are much more serious (14).

Seat belt use is lowest in city centres, as people often erroneously think that low speeds make seat belts unnecessary. However, one should take note of the fact that an adult is only able to resist the weight of his body using his arms and legs up to speeds of about 7 km/h. In a colliding vehicle travelling at 50 km/hour, the body is acted upon by forces similar to those that would occur in case of a fall from 10 m, and the forces only become greater with higher speeds. Studies emphasise different factors (15). In a Nigerian study (16), as much as 86% of respondents answered that the seat belt must be used during driving, while 95.5% of drivers said that they did not agree with the statement that the failure to use a seat belt may cause a traffic accident. In our own study, the share of drivers who always use the seat belt when driving in front increased.

In northern Italy, where traffic accident-related mortality is about 30% higher than the Italian average, the greatest risk factor for a fatal traffic accident is failure to use the seat belt. It is believed interventions in criminal prosecution, driver behaviour and the environment would be necessary to remedy the situation (17). Such measures have already proved effective in Slovenia, as indicated by the results of our study for individual years.

Research carried out in the United Arab Emirates found age, education level, gender, marital status and personal habits to have a significant effect, leading the researchers to believe measures should be implemented as part of traffic safety campaigns to raise awareness regarding seat belt use (18).

Spanish drivers believe that higher speeds on highways are associated with higher risk and that seat belts can provide protection in this regard. Failure to use a seat belt is associated with driving safety awareness, discomfort, social status (low level of education) and distrustfulness regarding seat belt effectiveness. Fear of punishment for failure to use the seat belt is not considered significant. They conclude that this necessitates preventive education [social pressure] (19). Among young drivers, failure to use the seat belt is among the most significant risk factors for having a traffic accident (20). A driver's specific behaviour, consumption of alcohol or drugs and failure to use the seat belt all significantly increase the probability of major traffic accidents and injuries.

Driving mistakes tend to have less of an effect than the driver's personal characteristics, while age and gender are almost negligible as factors (21). In the United States of America, research has been carried out into the influence of various cultural variables on seat belt use. It has been discovered that religion, race and political orientation all have a positive effect, while the effects of income and education were negligible, indicating new possibilities, in education and legislation, to achieve higher seat belt use and consequently better traffic safety (22). Findings of the US study show that failure to use the seat belt is associated with antisocial behaviour and coincident psychological distress. Among those least likely to use the seat belt are younger men, those with low income and those with higher education. People are also less likely to use the seat belt as passengers in a car if they are under the influence of alcohol or other drugs or if they have committed any offences indicating antisocial behaviour or criminal offences (23).

Among professional drivers of delivery vehicles, as much as 67% answered that they always use the seat belt (which only 31% of other drivers do). The common reasons for failing to use it are: frequent stops (29%) and ignorance of the risks involved (23%). Seat belt use is highest among delivery vehicle drivers (88%) followed by bus drivers (87%). On the other hand, seat belt use is only 60% among truck drivers (5, 24).

A study in Qatar has determined as much as 23% of the drivers who had caused a traffic accident were not using seat belts at the time, while causes of traffic accidents were determined to be dominated by the human factor (25). Research in Iran has also determined that the level of seat belt use in cars is very low, prompting researchers to propose development and implementation of effective intervention measures in order to encourage seat belt use (26).

The greatest part of those injured because of a failure to use the seat belt is made-up by young male drivers and those driving under the influence of alcohol, involved in single-car accidents on rural roads. Twenty per cent of drivers injured in traffic accidents were not wearing seat belts, while their share among fatalities was as high as 68%. Non-users involved in traffic accidents are at a significantly higher risk for serious injuries of the head, face, chest, abdomen and lower limbs (27). A study carried out in Boston has found the rate of seat belt use to be about 80% at the national level, but only around 63% in Massachusetts. Less likely to use a seat belt were men,

those who consume alcohol (over five drinks in a single episode), those who drink during driving and those who responded that they are bothered by the seat belt or had forgotten to fasten it (28). A Greek study has found seat belt use in cities to be lower than in the country, and that young and elderly male drivers were least likely to buckle-up. Seat belts were especially rarely used by back-seat passengers (29). The results in our study were similar. In 2000, shares of drivers who did not use their seat belts among all traffic accident fatalities were as follows: 39% in Finland, 31% in Germany, 30% in Ireland, 50% in Austria, 39% in Spain and 42% in Greece (30). The past few decades have seen seat belt use increase everywhere, including the most developed countries; in the US, for example, the increase was from 27% in 1985, through 49% in 1990, 68% in 1995, 71% in 2000, 82% in 2005, to 93% in 2010 (31).

CONCLUSION

We can conclude seat belt use among adult citizens of Slovenia is on the rise both in the front and in the back of the car, with the highest increase seen in front seats. We will thus have to continue implementing existing activities intended to raise awareness and seat belt use. Further data will have to be obtained in order for us to be able to exactly evaluate behaviour-affecting risk factors (*eg*, psycho-physical wellbeing, use of medicinal products).

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REFERENCES

1. Shibata A, Fukunda K. Risk factor of fatality in motor vehicle traffic accidents. Accident Anal Prev 1994; **26:** 391–7.
2. Bradbury A, Robertson C. Prospective audit of the pattern, severity and circumstances of injury sustained by vehicle occupants as a result of road traffic accidents. Arch Emerg Med 1993; **10:** 15–23.
3. Evans L. The effectiveness of safety belts in preventing fatalities. Accident Anal Prev 1986; **18:** 229–41.
4. Shinar D, Schechtman E, Compton R. Self-report of safe driving behaviors in relationship to sex, age, education and income in US adult driving population. Accident Anal Prev 2001; **33:** 111–6.
5. Kim S, Kim K. Personal, temporal and spatial characteristics of seriously injured crash-involved seat belt non-users in Hawaii. Accident Anal Prev 2003; **35:** 121–30.
6. Official Journal of the Republic of Slovenia [Internet]. Road Traffic Rules Act (OJ RS, 35/2010) (in Slovene) [3 Dec 2014]. Available from: http://www.uradni-list.si/1/content?id=101702
7. Mcllvenny S, Al Mahrougi F, Al Busaidi T, Al Nabhani A, Al Hikmani F, Al Kharousi Z et al. Rear seat belt use as an indicator of safe road behaviour in a rapidly developing country. JR Soc Promo Health 2004; **124:** 280–93.
8. Williams EW, Reid M, Lindo JL, Williams JJ, French S, Singh P et al. Association between exposure/non-exposure to the mandatory seat belt law with regards to compliance in vehicle accident victims—a hospital review. W Indian Med J 2007; **56:** 236–49.
9. Shults RA, Elder RW, Sleet DA, Thompson RS, Nichols JL. Primary enforcement seat belt laws effective even in the face of rising belt use rates. Accident Accident Anal Prev 2004; **36:** 491–503.
10. Silveira AJ. Seat belt use in Argentina—a 10 year struggle. Traffic Inj Prev 2003; **4:** 173–85.
11. NETS, NHTSA, OSHA [Internet]. Guidelines for Employees to Reduce Motor Vehicle Crashes OSHA [12 Dec 2012]. Available from: http://www.osha.gov/Publications/motor_vehicle_guide.pdf
12. Zupančič A [Internet]. The collision at 50 km per hour as a drop of 10 meters deep (in Slovene), [15 Dec 2012]. Available from: http://www.dnevnik.si/magazin/svet-vozil/1042551997
13. Roškar, B. Seatbelt (in Slovene). Diploma work, Univerza v Mariboru, Fakulteta za varnostne vede; 2010.
14. Kmetič F [Internet]. The safety belt solves—if it is attached (in Slovene), [12 Dec 2012]. Available from: http://www.motorevija.si/si/345/1484/Varnostni_pas_resuje_ce_je_pripet.aspx
15. Institute of safe way [Internet]. Safety in traffic (in Slovene), [6 Feb 2007]. Available from: http://www.varna-pot.si/default.aspx.
16. Onyema CR, Oladepo O. Knowledge and attitude of safety belt use among professional drivers in a tertiary Nigerian institution. Int J Inj Contr Saf Promot 2011; **18:** 57–64.
17. Valent F, Schiava F, Savonitto C, Gallo T, Brusaferro S, Barbone F. Risk factors for fatal road traffic accidents in Udine, Italy. Accid Anal Prev 2002; **37:** 71–84.
18. Bendak S, Al-Saleh K. Seat belt utilisation and awareness in UAE. Int J Inj Contr Saf Promot 2013; **20:** 342–8.
19. Cunill M, Gras ME, Planes M, Oliveras C, Sullman MJ. An investigation of factors reducing seat belt use amongst Spanish drivers and passengers on urban roads. Accid Anal Prev 2004; **36:** 439–45.
20. Ferguson SA. Other high-risk factors for young drivers—how graduated licensing does, doesn't, or could address them. J Safety Res 2003; **34:** 71–7.
21. Kim K, Nitz L, Richardson J, Li L. Personal and behavioral predictors of automobile crash and injury severity Accid Anal Prev 1995; **27:** 469–81.
22. Molnar LJ, Eby DW, Dasgupta K, Yang Y, Nair VN, Pollock SM. Explaining state-to-state differences in seat belt use: a multivariate analysis of cultural variables. Accid Anal Prev 2012; **47:** 78–86.
23. Vaughn MG, Salas-Wright C, Piquero AR. Buckle up: non-seat belt use and antisocial behavior in the United States. An Epidemiol 2012; **22:** 825–31.
24. Kim M, Yamashita EY. Attitudes of commercial motor vehicle drivers towards safety belts. Accid Anal Prev 2007; **39:** 1097–106.
25. Burgut HR, Bener A, Sidahmed H, Albuz R, Sanya R, Khan WA. Risk factors contributing to road traffic crashes in a fast-developing country: the neglected health problem. Ulus Travma Acil Cerrahi Derg 2010; **16:** 497–502.
26. Ali M, Haidar N, Ali NM, Maryam A. Determinations of seat belt use among drivers in Sabzevar, Iran: a comparison of theory of planned behavior and health belief model. Traffic Inj Prev 2011; **12:** 104–9.
27. Allen S, Zhu S, Sauter C, Layde P, Hargarten S. A comprehensive statewide analysis of seat belt non-use with injury and hospital admissions: new data, old problem. Acad Emerg Med 2006; **13:** 427–34.
28. Fernandez WG, Mehta SD, Coles T, Feldman JA, Mitchell P, Olshakr J. Self-reported safety belt use among emergency department patients in Boston, Massachusetts. BMC Public Health 2006; **6:** 111–21.
29. Yannis G, Laiou A, Vardaki S, Papadimitriou E, Dragomanovits A, Kanellaides G. Parameters affecting seat belt use in Greece. Int J Contr Saf Promot 2011; **18:** 189–97.
30. ETSC [Internet]. Seat Belt Reminders, [2 Dec 2006]. Available from: http://archive.etsc.eu/documents/ETSC_Seat_belt_reminder_oct_06.pdf

31. NHTSA [Internet]. National Highway Traffic Safety Administration, US Department of Transport NHTSAs Depr C Stat Admin, [2 Dec 2012]. Available from: http://www.nhtsa.gov/

ORIGINAL ARTICLE

Knowledge, Attitude and Beliefs of Undergraduate Medical and Dental Students towards Dental Treatment during Pregnancy

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ABSTRACT

Objective: *To determine the knowledge, attitudes and beliefs of pre-clinical medical and dental undergraduate students, at the University of the West Indies, towards dental treatment during pregnancy.*

Methods: *All 1st and 2nd year students attending the University of the West Indies, Schools of Dentistry and Medicine were invited to participate in a self-administered validated questionnaire which was piloted on the dental interns for item clarity.*

Results: *Two hundred and seventy-seven students participated in the study. Mean age 20.6 years, 53.8% female, with the major ethnic groups showing 56.2% Indo-Trinidadian and 21% Mixed. Some (12.3%) thought that swollen gums were associated with pregnancy, while 28% of participants felt that bleeding gums were not associated with pregnancy. The majority of participants (61.8%) felt that it was safe to conduct dental examinations during pregnancy, however, 27.6% of the participants felt that radiographs were safe during pregnancy. The majority (54%) were uncertain whether the pregnancy was associated with tooth decay. More than three-quarter of the students (77.9%) were unsure whether oral disease was associated with pre-eclampsia.*

Conclusion: *These data provide the first insight into the knowledge, attitudes and beliefs of pre-clinical dental and medical undergraduate students on pregnancy and oral health in the Caribbean. The knowledge of the participants in this study was low, which underscores the need to educate all future health professionals at the pre-clinical level on the correlation between dental health and pregnancy and the importance of the effects of dental treatment on systemic diseases.*

Keywords: Pregnancy, undergraduate, West Indies.

INTRODUCTION

Several studies have shown that there is an association between maternal oral health and the general health of both the pregnant woman and her child (1). Pregnancy is associated with an increased risk of oral conditions such as dental caries, pyogenic granulomas, mobile teeth, gingivitis and periodontitis (2). The incidence of periodontal disease in pregnant women is higher than in non-pregnant women, possibly due to the increase in the concentration of oestrogen and progesterone during pregnancy.

Periodontal disease is an inflammatory response, by the host to bacterial infection, which leads to the destruction of the bone and surrounding tissues (3). It is a common chronic infection that has a variable prevalence, between 35% and 79%, worldwide (4). Periodontal disease has recently been suggested as a contributory factor to impaired glucose metabolism in women who had a history of gestational diabetes (5). Periodontitis has been associated with poor pregnancy outcomes such as preterm birth, low birth weight and gestational diabetes.

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Moreover, it was suggested that periodontal disease may increase the risk of having preterm low birth weight infants. This is as a result of the biologic mediators of inflammatory processes such as prostaglandins E2 and tumour necrosis factor. The common bacterial product lipopolysaccharide may also have a triggering role which adversely changes the course of pregnancy (5).

Many women do not present for dental care during their pregnancy even though all dental prevention, diagnosis and treatment can be safely provided during the entire pregnancy (1). This may be due to the dental practitioner's and the mother's knowledge and beliefs about dental care during pregnancy (1). In a study on medical doctors' attitudes and knowledge of the association between oral health and pregnancy outcomes, it was shown that physicians do not routinely advise their patients to seek dental care during pregnancy (6).

In general, little is known about health care professionals' knowledge and attitudes regarding oral health care during pregnancy (7), and to the best of our knowledge, there is no such information for undergraduate dental and medical students. Therefore, the knowledge, attitudes and beliefs of dental and medical students are important to decrease the paucity of information on this topic.

SUBJECTS AND METHODS

The sampling frame for this study comprised the class register for all 1st and 2nd year students attending the University of the West Indies (UWI), Schools of Dentistry and Medicine, and these students were invited to participate in a self-administered questionnaire consisting of 26 questions. Those who were in clinical years three to five or were graduates of the UWI School of Dentistry and School of Medicine or were under 18 years old were excluded from the study.

Undergraduate, pre-clinical students in 1st and 2nd years of medicine and dentistry were asked by six dental students of 2nd year to participate in the questionnaire prior to their didactic lectures at the Faculty of Medical Sciences. The reason for non-participation was not ascertained.

The questionnaire was based on a validated one used in a similar study on Obstetricians (8) and was piloted on the dental interns at the School of Dentistry for item clarity. The questionnaire was divided into three sections:

(1) The students' demographics, medical or dental year of undergraduate study;
(2) The students' knowledge of associations between pregnancy and oral health;
(3) The students' knowledge of oral health care during pregnancy and attitudes towards oral healthcare during pregnancy.

In sections two and three the responses were yes, no and don't know (Table 1). Ethical approval for the study was given by the University of the West Indies Research Ethics Committee and written consent for the study was obtained from each student. Data collection took place between January and March 2015. Data were analysed using SPSS version 22.0.

Table 1: First- and second-year dental and medical students' responses on the effects of oral disease on pregnancy outcomes, pregnancy effects on oral health and safe oral health interventions in pregnancy

Effect of pregnancy on oral health	Yes (%)	No (%)	Unsure (%)
Swollen gums	12.3	19.9	67.9
Bleeding gums	11.3	28.0	60.7
Excess tooth decay	16.3	29.7	54.0
Tooth loss	10.5	38.6	50.9
Effect of oral disease on pregnancy outcomes			
Preterm birth	10.9	21.1	68.0
Low birth weight	17.3	20.6	62.1
Abortion	4.7	35.4	59.9
Still birth	5.4	31.8	62.8
Pre-eclampsia	6.5	15.6	77.9
Safe oral health interventions during pregnancy			
Dental examination	61.8	10.5	27.6
X-ray safety	27.6	21.5	50.9
Dental extraction	26.2	28.7	45.1
Tooth brushing	89.5	1.4	9.1
Flossing	89.8	3.6	6.5
Mouth wash	76.1	8.0	15.9
Local anaesthetic	16.3	33.3	50.0
Antibiotics	13.0	44.9	42.0
Would recommend a dental visit during pregnancy	87.0	12.7	0.4
Would recommend a delayed dental visit during pregnancy	19.6	80.4	–
Attitudes of students towards oral treatment during pregnancy			
Examination	95.3	4.7	–
Routine cleaning	90.5	9.5	–
Periodontal cleaning	76.6	23.4	–
Fillings/crowns	47.6	52.4	–
Extractions	30.4	69.6	–
Students' sources of information			
Book/magazine/pamphlet	21.1	78.9	–
Medical/dental journal	16.2	83.8	–
Undergraduate curriculum	17.8	82.2	–
TV	32.4	67.6	–
Internet	42.7	57.3	–
Other	59.3	40.7	–

RESULTS

The reasons for non-response was not ascertained in our study; however, the majority (68%) of non-responders was from the medical undergraduates in year two.

Knowledge of associations between pregnancy and oral health

Some (12.3%) participants thought that swollen gums were associated with pregnancy, while some of the participants (28%) felt that bleeding gums were not associated with pregnancy (Table 1).

Knowledge of associations between oral health and pregnancy outcomes

There was a general lack of knowledge by the undergraduate students with respect to the effect of oral disease on pregnancy outcomes; few (10.9%) thought that oral disease was associated with preterm birth (Table 1).

Beliefs of safe oral health interventions during pregnancy

The majority of participants (61.8%) felt that it was safe to conduct dental examinations during pregnancy, while 27.6% felt that radiographs were safe during pregnancy. The largest proportion (45.1%) did not know if extractions were safe during pregnancy, while 33.3% felt that local anaesthesia was unsafe.

Characteristics of the participants

Two hundred and seventy-seven students participated in this study, 160 students of 277 students in the 1st year and 117 students of 316 students in the 2nd year of medicine and dentistry participated in the study. There was a response rate of 46.7%. The majority of students (80.5%) were medical undergraduate students. The participants were aged between 18 and 30 years with a mean age of 20.6 (SD = 2). Most participants (53.8%) were female; the main ethnic groups comprised Indo-Caribbean (56.2%), Mixed (21 %) and Afro-Caribbean (15.2%) (Table 2).

Attitudes towards oral healthcare during pregnancy

Most of the participants (87.4%) stated that they would recommend a dental visit during pregnancy, while 16.5% said they would delay this dental visit. The majority (88.3%) would advise their patients to have routine cleanings during pregnancy; however, some (68%) would not advise their pregnant patients to have dental extractions (Fig. 1).

Table 2: Characteristics of the participants

Undergraduate programme	Frequency	Valid percentage (%)
Dentistry	54	19.5
Medicine	233	80.5
Age		
18–24	261	94.6
25–30	15	5.4
Gender		
Male	128	46.2
Female	149	53.8
Year of Study		
1st	160	57.8
2nd	117	42.2
Ethnicity		
Afro Caribbean	42	15.2
Indo Caribbean	155	56.2
Caucasian	9	3.2
Chinese	1	0.4
Mixed	58	21.0
Other	11	4.0

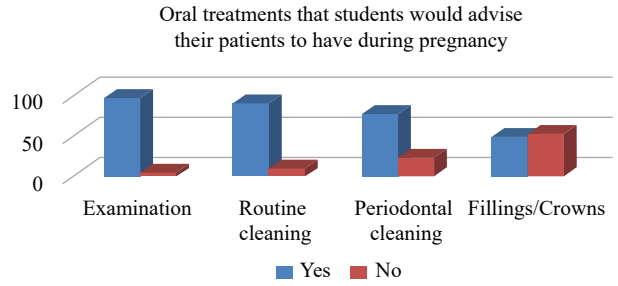

Figure: Attitudes towards oral healthcare during pregnancy.

DISCUSSION

During pregnancy there is an increased response to dental plaque which leads to swollen gingivae which are prone to bleeding on brushing (9). In a recent study on dental interns (10), most of their participants (92%) agreed that pregnancy increased the tendency to have gingival inflammation, while in our study only 12.3% thought that pregnancy was associated with swollen and 11.3% thought that pregnancy was associated bleeding gums.

Given that periodontal disease may affect pregnancy outcomes, one study suggests that dentists should play a proactive role in the maintenance of oral health of pregnant women (11). In our study, the majority of participants (87%) stated that they would recommend dental visits and 80.4% would not recommend that the dental visit be delayed during pregnancy. This is a positive finding. In another study on Brazilian public health professionals (7), the authors opined that health care

professionals who had more favourable attitudes had a more positive approach to understanding the connection between oral and systemic health.

Pregnancy does not lead to tooth loss; however, only 38.6% of the participants in this study shared this belief, which is in contrast to a study on Obstetricians, in which the majority of their respondents correctly responded to this belief. Patients who are pregnant often pay visits to the dentist for tooth related pain and infections. These conditions often require the use of a diagnostic imaging technique to aid in their management. In a study on dental interns (10), more than half (63%) said that there were no problems with X-rays during pregnancy and only 9% did not know, whereas in our study only 27.6% stated that X-rays were safe and more than half of the students (50.9%) were unsure.

This finding is of interest because the delay of dental treatment during pregnancy due to the postponement of dental radiography may have an adverse effect on mother and foetus. Although X-rays are a form of ionising radiation that can result in cell and DNA damage, it has been found that dental radiation exposure to the foetus is negligible (12, 13). The current recommendations are that routine dental radiographs should be avoided during all trimesters of pregnancy but they can and should be used selectively whenever needed. All of the various techniques for minimising the absorbed radiation dose like rectangular collimation of the beam and the use of the lead apron should be undertaken when radiographs are necessary in the pregnant patient. Thus, education of students about the safety of dental radiation during pregnancy plays an integral role in the future health and well-being of their pregnant patients and their unborn children.

Additionally, only 16.3% of the dental students who participated in our study thought local anaesthetic was a safe intervention during pregnancy, which was much less when compared to another study where 60% of their dental interns thought that it can be used (10). This is a negative conclusion with respect to our research, as it was found in a study on the oral health care of patients (11) that local anaesthetic can be safely administered to pregnant patients, who are healthy, once proper aspiration technique and correct dosages are used. In our study, the major response to the safety of antibiotics during pregnancy was that it was not safe (44.9%) or many (42%) were unsure, whereas Amoxicillin was the most commonly prescribed antibiotic of dental interns (96%) in their study (10).

In this study, the vast majority of participants (61%) correctly believed that examinations were safe during pregnancy and 95.3% had a positive attitude towards recommending that pregnant patients have dental examinations. This is a much higher than a similar study (6), where only 49% of medical doctors reported that they advised their pregnant patients to have dental visits and unfortunately 88% advised their patients to delay dental treatment until after pregnancy, whereas in our study only 19.5% had this advice. The undergraduate students also had positive attitudes towards routine dental cleanings, where 90.5% stated that they would advise this treatment during pregnancy and 76.6% would recommend periodontal cleanings.

The majority of the participants in this study did not know about the relationship between pregnancy and oral health and vice versa and their knowledge was in general poor, where only 10.9% correctly believed that oral disease was associated with preterm delivery and 6.5% with pre-eclampsia. In addition to this, 17.3% of students found that oral disease was correctly correlated with low birth weight. These results were in contrast to that of the study on Obstetricians in which the majority correctly stated that there was a link between the health of gums and pregnancy and periodontitis could eventually cause preterm birth (8). This contrast maybe attributed to the difference in the level of knowledge between undergraduate medical and dental students and Obstetricians.

The greatest proportion of students (59.3%) stated that their information on oral health and pregnancy was derived from a source other than what was listed in the questionnaire followed by the Internet (42.7%). These results were in contrast to the findings on medical doctors (6), in which 85% of medical doctors found books and magazines to be their source of learning this information and in another study on dental interns, their main source were books (61%) and (30%) Internet (10).

The University of the West Indies, Faculty of Medical Sciences, has shared classes for the medical and dental students in the 1st and 2nd years of undergraduate training. A recent study (7) showed that there should be encouraged opportunities for learning about oral and systemic health; especially since health care professionals who had worked in the public service or had post-grad programs were more favourable to oral health aspects of pregnancy. Moreover, in a study on the training of midwifery students in oral care of pregnant patients, they showed that the attitude of their participants improved three months after an intervention (14). It is therefore believed that this target group of preclinical undergraduate students

would be ideal to incorporate classes on the association between pregnancy and oral health.

The participants of this study are not representative of all pre-clinical medical and dental undergraduate students studying in Trinidad and the sample size is small.

CONCLUSION

These data provide the first insight into the knowledge, attitudes and beliefs of pre-clinical dental and medical undergraduate students on pregnancy and oral health in the Caribbean. The holistic approach of modern-day medicine gives a direct indication of the importance of correspondence between medicine and dentistry. Given that the knowledge of the participants in this study was low, our findings illustrate the need to educate not only dentists but also all future health professionals at the pre-clinical level on the correlation between dental health and pregnancy and the importance of the effects of dental treatment on systemic diseases.

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REFERENCES

1. Hughes D, Lee PR. Oral Health during Pregnancy and Early Childhood: Evidence-Based Guidelines for Health Professionals. CDA Foundation; 2010. At: http://www.cdafoundation.org/Portals/0/pdfs/poh_guidelines.pdf Accessed: September 6, 2015.
2. Silk H, Douglass A, Douglass MJ, Silk L. Oral health during pregnancy. AAFP 2008; **77:** 1139–44.
3. Bascones-Martínez A, Muñoz-Corcuera M, Noronha S, Mota P, Bascones-Ilundain C, and Campo-Trapero J. Host defence mechanisms against bacterial aggression in periodontal disease: basic mechanisms. Medicina Oral, Patologia Oral y Cirugia Bucal 2009; **14:** e680–85.
4. Alabander JM. Epidemiology and risk factors of periodontal disease. Dent Clin North Am 2005; **49:** 517–32.
5. Xiong X, Elkind-Hirsch KE, Xie Y, Delarosa R, Maney P, Pridjian G and Buekens P. Periodontal disease as a potential risk factor for the development of diabetes in women with a prior history of gestational diabetes mellitus. J Public Health Dent 2012; **73:** 41–9.
6. Al-Habashneh R, Aljundi SH, Alweli HA. Survey of medical doctors' attitudes and knowledge of the association between oral health and pregnancy outcomes. Int J Dent Hyg 2008; **6:** 214–20.
7. Alves RT, Ribeiro RA, Costa LR, Leles CR, Freire M do C M, Paiva SM. Oral care during pregnancy: attitudes of Brazilian Public Health professionals. Int J Environ Res Pub Health 2012; **9:** 3454–64.
8. Suri V, Rao NC, Aggarwal N. A study of obstetricians' knowledge, Attitudes and practices in oral health and pregnancy. Educ Health 2014; **27:** 51–4.
9. Pirie M, Cooke I, Linden G, Irwin C. Dental manifestations of pregnancy. TOG 2007; **9:** 21–6.
10. Aljulayfi I, Alrusayni A, Alqahtani S, Hamam M K. Awareness of dental interns in managing cases of pregnant women in Saudi Arabia. Saudi J Dent Res. 2015; **6:** 26–9.
11. Giglio JA, Lanni SM, Laskin DM. Oral health care for the pregnant patient. J Can Dent Assoc. 2009; **75:** 43–8.
12. Kusama T, Ota K. Radiological protection for diagnostic examination of pregnant women. Congenit Anom (khoto) 2002; **42:** 10–4.
13. Richards AG. Dental X-ray protection. Dent Clin North Am 1968: 631–41.
14. Mohebbi SZ, Yazdani R, Sargeran K, Tartar Z, Janeshin A. Midwifery students training in oral care of pregnant patients: an interventional study. J Dent (Tehran) 2014; **11:** 587–95.

ORIGINAL ARTICLE

Racial Differences in Seasonal Variation in Election and Non-election Years in the Male-to-Female Ratio at Birth in the United States

V Grech, T Borg

ABSTRACT

Objective: *In humans, male births exceed female births. This ratio is conventionally expressed as M/F and is influenced by a large number of factors, including stress. This study was carried out in order to ascertain whether the known seasonal variation in M/F in the United States of America (USA), peaking in June, is affected by the quadrennial elections (November), and whether any such influences vary by race.*

Methods: *Births by gender and by race for the period 2003–2013 were obtained from the website of the Centers for Disease Control and Prevention for the four available races: White, Black/African American, Asian/Pacific Islander and American Indian/Alaska Native. Election years were 2004, 2008 and 2012. Seasonality tests were carried out for the entire group and for White and Black/African American births.*

Results: *This study analysed 45 138 496 live births (23 102 106 males, 22 036 390 females, M/F: 0.51180). Overall, M/F was the lowest in election years, rising, then falling Asian/Pacific Islander births but not for Black/African American or American Indian/Alaskan Native births. Overall and for White births, only election year plus 3 (year just before election) showed seasonal variation (*$p < 0.01$*).*

Conclusion: *Seasonality may have been disturbed/reduced in most years due to the elections. Black births may have been unaffected due to chronic stress caused by socio-economic dampening of M/F trends.*

Keywords: Birth rate/trends, infant, newborn, periodicity, seasons, gender ratio, United States

INTRODUCTION

In humans, male births exceed female births and this ratio is conventionally referred to M/F, denoting male births divided by total births. The male-to-female births ratio (M/F) may be influenced by a large number of factors (1) and has been shown to exhibit seasonal variation in various parts of the world (2). Early studies had shown a low M/F in February and March and a high M/F in summer in various parts of the world (3) with similar patterns in the United States of America (USA) more recently (4).

The Trivers–Willard hypothesis proposes that individuals who are able to influence their offspring gender ratio in accordance with their environment are likelier to procreate, thereby dispersing these advantageous genes. In polygynous species, only the fittest males reproduce. For this reason, parental investment in a 'good-quality' son may yield greater numbers of descendants than an equivalent investment in a 'good-quality' daughter. It is thus advantageous for a mother to reproduce sons when she has good resources, and daughters when she does not. This is known as the Trivers–Willard hypothesis (5). For the same reasons, it has been postulated that the findings that stress reduces M/F may be explained by the Trivers–Willard hypothesis.

The above-mentioned seasonal patterns of live births support the Trivers–Willard hypothesis since the birth of offspring in favourable seasonal conditions increases the chances of said offsprings' survival. It has also been recently shown that in the USA, for the

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period 2003–2013, M/F was the highest in Asian/Pacific Islanders, followed by White, American Indian/Alaska Native and Black/African American births, with these differences occurring at statistically significant levels (4). Significant seasonality was present overall in the USA, with a peak in June, for Whites more than Black/African American, and absent in the rest. It was conjectured that the traditionally lower M/F found in Black/African and American Indian/Alaskan births may be stress related in that these races are overall socio-economically underprivileged and hence chronically stressed (6). It was therefore also hypothesised that the dampened seasonality noted in Black/African American births might also be due to this phenomenon (4).

In the USA, election day is set by the law for the general election of public officials. The event and the associated political campaigns have been shown to engender significant stress in the populace (7, 8). Election day always occurs on the Tuesday after the first Monday in November (November 2nd to the 8th) and the Presidential Elections occur every four years. This provides a unique opportunity to ascertain whether the election campaigns and the elections themselves exert any influence on M/F (9).

This study was carried out in order to ascertain whether the seasonal variation in M/F in the USA is influenced by the Presidential Elections and whether there are any racial variations.

SUBJECTS AND METHODS

Data and definitions

Births by gender and by race for the period 2003–2013 were obtained from the website of the Centers for Disease Control and Prevention in the CDC Wonder section (http://wonder.cdc.gov/natality.html). The data was available for four races: White, Black/African American, Asian/Pacific Islander and American Indian/Alaska Native. Ethical approval was irrelevant as this analysis comprised a large and completely anonymous dataset. For the same reason, informed consent was unnecessary.

Election years were 2004, 2008 and 2012. Election years plus one were 2005, 2009 and 2013. Election years plus two were 2006 and 2010. Election years plus three (pre-election years) were 2003, 2007 and 2011. Seasonality tests were carried out on the above-mentioned year groups for all Americans, White Americans and Black/African Americans. American Indian/Alaskan Native and Asian or Pacific Islander were not analysed for seasonality due to the (relatively) small numbers of these births.

Statistical analysis

Prior to any use of statistical tools, a series of tests were done on the data to check for homogeneity of variance, independence of observations and normality. Data were also plotted to check for obvious outliers.

Seasonality was analysed using Demetra (version 1.0.4.323) and a model-based method (X12) was operated to fit an Autoregressive Integrated Moving Average (ARIMA) model to the data. A series of seasonality tests were carried out on each time series after the ARIMA model was determined. These included non-parametric tests for stable seasonality using the Friedman and Kruskall–Wallis tests, a test for the presence of seasonality assuming stability, evolutive seasonality test and combined seasonality test. The combined seasonality test passes if the first three tests pass at the 1% ($p < 0.01$) level and if the evolutive seasonality test fails at the 20% ($p > 0.2$) level.

Further tests on seasonality using analysis of variance (ANOVA) were carried with the null hypothesis showing no statistically significant difference between the means of each month. An ANOVA was carried out with Statistical Package for the Social Sciences (SPSS, International Business Machines Corporation, New York, USA). A p-value < 0.05 was taken to represent a statistically significant result.

The quadratic equations of Fleiss were used to calculate the exact 95% confidence limits (10). Chi tests were used for trend testing of male and female births using the Bio-Med-Stat Excel add-in for contingency tables (Peter Slezák, Bratislava, Slovakia) (11).

RESULTS

This study analysed a total of 45 138 496 live births as 23 102 106 males and 22 036 390 females (M/F: 0.51180, 95% CI: 0.51166, 0.51195) born over the period 2003–13. Annual totals by race for election years (2004, 2008, 2012), election years plus one (2005, 2009, 2013), election years plus two (2006, 2010) and election years plus three (pre-election years: 2003, 2007, 2011) are shown in Table 1.

Overall, M/F was the lowest in the election years, rising then falling again in the election year. This pattern was present for White and Asian/Pacific Islander births but not for Black/African American or American Indian/Alaskan Native births. These trends were not statistically

Table 1: Annual totals by race and male-to-female birth ratios for amalgamated years (election, election +1, +2 and +3)

	Election year	Election year + 1	Election year + 2	Election year + 3	Total
American Indian/Alaskan Native					
Male	70 992	71 034	47 981	70 925	260 932
Female	68 565	68 435	46 500	67 989	251 489
Total	139 557	139 469	94 481	138 914	512 421
UCL	0.51132	0.51194	0.51103	0.51320	0.51058
M/F	0.50870	0.50932	0.50784	0.51057	0.50921
LCL	0.50607	0.50669	0.50464	0.50794	0.50784
Asian or Pacific Islander					
Male	388 955	385 772	252 057	376 193	1 402 977
Female	366 155	362 098	235 874	353 413	1 317 540
Total	755 110	747 870	487 931	729 606	2 720 517
UCL	0.51622	0.51696	0.51799	0.51676	0.51630
M/F	0.51510	0.51583	0.51658	0.51561	0.51570
LCL	0.51397	0.51469	0.51518	0.51446	0.51511
Black or African American					
Male	976 945	977 766	663 794	970 152	3 588 657
Female	944 064	947 746	639 112	938 272	3 469 194
Total	1 921 009	1 925 512	1 302 906	1 908 424	7 057 851
UCL	0.50927	0.50850	0.51033	0.50906	0.50883
M/F	0.50856	0.50780	0.50947	0.50835	0.50846
LCL	0.50785	0.50709	0.50861	0.50764	0.50809
White					
Male	4 862 592	4 811 220	3 267 340	4 908 388	17 849 540
Female	4 634 319	4 577 124	3 112 283	4 674 441	16 998 167
Total	9 496 911	9 388 344	6 379 623	9 582 829	34 847 707
UCL	0.51234	0.51279	0.51254	0.51252	0.51238
M/F	0.51202	0.51247	0.51215	0.51221	0.51222
LCL	0.51170	0.51215	0.51176	0.51189	0.51205
All					
Male	6 299 484	6 245 792	4 231 172	6 325 658	23 102 106
Female	6 013 103	5 955 403	4 033 769	6 034 115	22 036 390
Total	12 312 587	12 201 195	8 264 941	12 359 773	45 138 496
UCL	0.51191	0.51218	0.51228	0.51207	0.51195
M/F	0.51163	0.51190	0.51194	0.51179	0.51180
LCL	0.51135	0.51162	0.51160	0.51152	0.51166

UCI = upper confidence interval; LCI = lower confidence interval.
Election years: 2004, 2008 and 2012
Election years plus one: 2005, 2009 and 2013.
Election years plus two: 2006 and 2010.
Election years plus three (pre-election years): 2003, 2007 and 2011.

significant, even when data were pooled for White and Asian/Pacific Islander births.

Monthly M/F for the entire period is shown in Fig. 1. The same data for election years (2004, 2008 and 2012), election years plus one (2005, 2009 and 2013), election years plus two (2006 and 2010) and election years plus three (pre-election years: 2003, 2007 and 2011) are shown in Figs. 2–5, respectively.

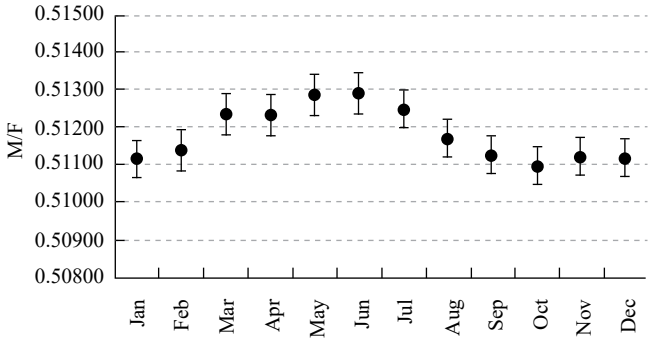

Fig. 1: Monthly M/F for 2003–13.
M/F = male-to-female ratio.

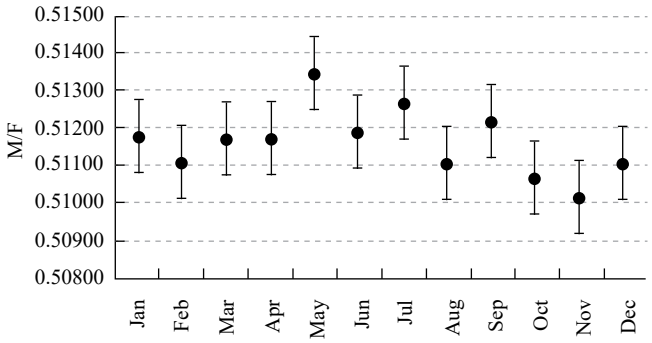

Fig. 2: Monthly M/F for election years (2004, 2008 and 2012).
M/F = male-to-female ratio.

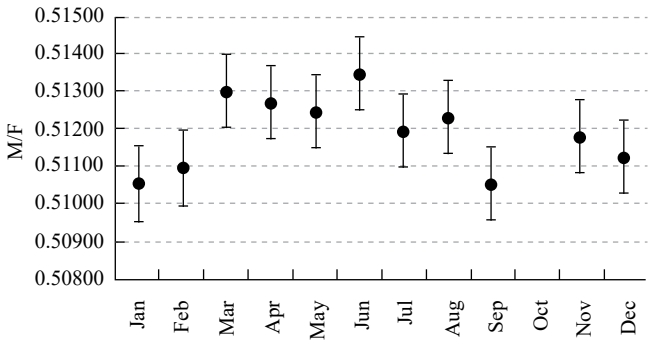

Fig. 3: Monthly M/F for election years plus one (2005, 2009 and 2013).
M/F = male-to-female ratio.

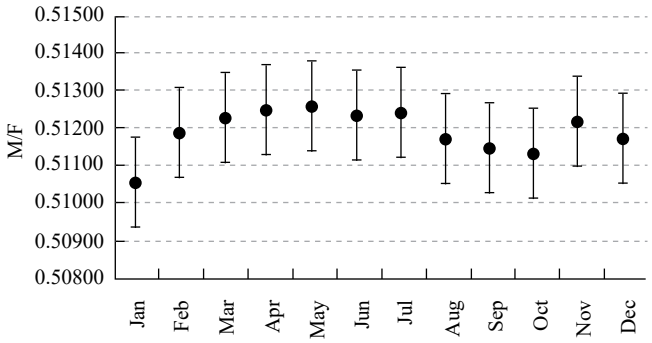

Fig. 4: Monthly M/F for election years plus two (2006 and 2010).
M/F = male-to-female ratio.

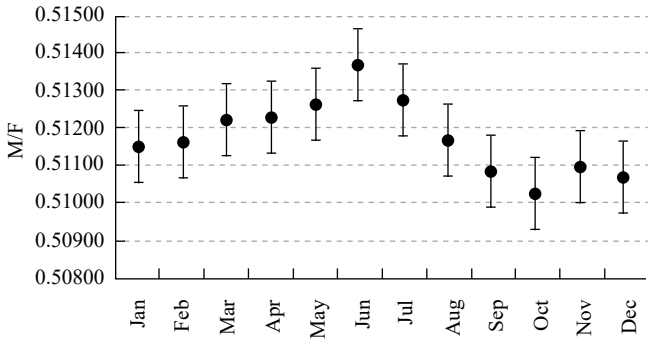

Fig. 5: Monthly M/F for election years plus three (pre-election years: 2003, 2007 and 2011).
M/F = male-to-female ratio.

The ARIMA (0, 1, 1) (0, 1, 1) was fitted on all data and for three of the four time series being analysed. For the total American population, that is, including all four races, of all four time series, election year plus three exhibited a seasonal variation at $p < 0.01$. Combined seasonality was absent in election years and election years plus one (Table 2). Nevertheless, ANOVA results show significant differences between months at $p < 0.05$ for all years under the study.

For election years plus two (2006 and 2010), the time series was too short for the ARIMA modelling. Additionally, the data violated the assumptions for ANOVA testing. The Kruskall–Wallis test was used instead to check for seasonality for summated quarterly rather than monthly data. Seasonality was not present at even for quarterly data.

White American births exhibited the same seasonal pattern as total Americans, with seasonal variation present in the election years plus three (time series at $p < 0.01$). Similarly, ANOVA results for White American births confirmed the seasonality pattern for the time series grouping mentioned earlier. Seasonality was absent in Black/African American births for all time-series combinations under the study. For the other two races (Asian/Pacific Islander and American Indian/Alaska Native), the dataset was too small to attempt to perform seasonality analysis.

DISCUSSION

Overall

A previous study has shown significant seasonality in the USA overall, with an M/F peak in June (4). For the total population (all four races), the ANOVA showed significant differences between months at $p < 0.05$ for all years under the study.

Table 2: ANOVA and five seasonality test results on all four American races, and on separately on White and Black/African births

	ANOVA and five seasonality tests	All races	Whites	Black/
African				
All years	ANOVA	< 0.0001	< 0.0001	0.005
	Friedman test	< 0.0001	< 0.0001	0.0005
	Kruskall–Wallis test	< 0.0001	< 0.0001	0.0006
	Test for presence of seasonality assuming stability	< 0.0001	< 0.0001	0.0009
	Evolutive seasonality test	0.7993	0.4233	0.4649
	Combined seasonality test	SP	SP	SP
Election years (2004, 2008 and 2012)	ANOVA	0.001	0.006	0.533
	Friedman test	0.006	0.0191	0.3142
	Kruskall–Wallis test	0.0195	0.0316	0.3944
	Test for presence of seasonality assuming stability	0.001	0.0073	0.4215
	Evolutive seasonality test	0.9973	0.7674	0.1174
	Combined seasonality test	SA	SA	SA
Election years plus one (2005, 2009 and 2013)	ANOVA	0.01	0.008	0.109
	Friedman test	0.0069	0.0027	0.1986
	Kruskall–Wallis test	0.0243	0.0322	0.1233
	Test for presence of seasonality assuming stability	0.0032	0.0032	0.103
	Evolutive seasonality test	0.4851	0.5399	0.254
	Combined seasonality test	SA	SA	SA
Election years plus three (2003, 2007 and 2011)	ANOVA	0.0001	0.001	0.211
	Friedman test	0.0013	0.0002	0.1216
	Kruskall–Wallis test	0.0081	0.0063	0.1576
	Test for presence of seasonality assuming stability	0.0001	0.0001	0.1209
	Evolutive seasonality test	0.8634	0.7954	0.4627
	Combined seasonality test	SP	SP	SA

SA = seasonality absent; SP = seasonality present.
The Kruskall–Wallis test was used for election years plus two (2006 and 2010).

However, analysis by amalgamated years showed that only election years plus three exhibited a significant seasonal variation. It may be hypothesised that seasonality

was absent and therefore possibly disturbed or reduced by stress engendered by the electoral campaigns in election years and election years plus one. However, it is difficult to extend this explanation to election years plus two. A possible explanation for this is a type 2 error, since there were less data (one less year) in this group.

This socio-economic dampening effect on M/F is supported by the observed rise in M/F in what may be a recovery from stress after election years and a decline back down as the elections approach, since this is only observed in the more socio-economically privileged groups, White and Asian/Pacific Islander, but not for Black/African American or American Indian/Alaskan Native births.

Racial differences

A previous study had also shown significant seasonality in M/F for Whites more than Black/African American, and absent seasonality in the rest (4). It had also been shown that M/F was significantly highest in Asian/Pacific Islander ($p < 0.0001$), followed by White ($p = 0.002$), American Indian/Alaska Native ($p = 0.04$) and Black/African American births ($p = 0.04$) (4).

In this study, White American births exhibited the same seasonal pattern as the above-mentioned totals. This was the largest population and hence the group least likely to result in a type 2 error.

Seasonality was absent in Black/African American births for all time series combinations. This may be due to the fact that Black births were far less than White births, and therefore the possibility of a type 2 error cannot be discounted. However, it is possible that seasonality is truly absent in Black births since both ANOVA and combined seasonality testing indicate absence of seasonality. This may be because, as already alluded to, this population is chronically stressed due to socio-economic circumstances. Seasonality may therefore be dampened, and M/F effects due to election campaigns/elections may therefore not be manifest.

AUTHORS' NOTE

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REFERENCES

1. James WH. The human sex ratio. Part 1: a review of the literature. Hum Biol 1987; **59:** 721–52.
2. Melnikov V. Seasonal inconstancy of human sex ratio at birth. Early Hum Dev 2015; **91:** 817–21.
3. Huntington E. 1938. Season of Birth. New York: John Wiley and Sons, 1938.
4. Grech V, Borg T. Seasonal variation by race in the male to female ratio at birth in the United States. West Indian Med J. 2021; **69:** 129–33.
5. Trivers RL, Willard DE. Natural selection of parental ability to vary the sex ratio of offspring. Science 1973; **179:** 90–2.
6. Grech V. State and regional differences in the male-to-female ratio at birth in the United States, 1995-2012. West Indian Med J. 2015; **65:** 180–84
7. Stanton SJ, Beehner JC, Saini EK, Kuhn CM, Labar KS. Dominance, politics, and physiology: voters' testosterone changes on the night of the 2008 United States presidential election. PLoS One 2009; **4:** e7543.
8. Stanton SJ, Labar KS, Saini EK, Kuhn CM, Beehner JC. Stressful politics: voters' cortisol responses to the outcome of the 2008 United States Presidential election. Psychoneuroendocrinology 2010; **35:** 768–74.
9. Timpone RJ. Structure, behavior, and voter turnout in the United States. Am Polit Sci Rev 1998; **92:** 145–58.
10. Fleiss JL. Statistical methods for rates and proportions. 2nd ed. New York: John Wiley and Sons; 1981: 14–15.
11. Slezák P. Microsoft Excel add-in for the statistical analysis of contingency tables. Int J Innovation Educ Res 2014; **2:** 90–100.

ORIGINAL ARTICLE

Ameliorative Effect of Zinc Oxide and Silver Nanoparticles on Antioxidant System in the Brain of Diabetic Rats

M Afifi[1,2], AM Abdelazim[2]

ABSTRACT

Objective: *To test the ability of both zinc oxide (ZnONPs) and silver (SNPs) nanoparticles to ameliorate the oxidative stress resulting from diabetes in diabetic rats.*

Methods: *Fifty male albino rats were randomly used: 10 served as a control and 40 rats were injected with single dose (100 mg/kg) of streptozotocin (STZ) intraperitoneally. They were subdivided into untreated diabetic, + ZnONPs, diabetic + SNPs, and diabetic + insulin. The activities and messenger RNA (mRNA) expression levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRD) were determined in the brain tissues. Malondialdehyde, total antioxidant capacity (TAC), zinc (Zn) and silver (Ag) concentrations were estimated in the brain tissues of all rats.*

Results: *A significant increase in the activities and mRNA expression levels of SOD, CAT, GPx and GRD was shown. Malondialdehyde levels were significantly decreased where there was a significant increase in the Zn, Ag and TAC in the brain of ZnONP- and SNP-treated rats, when compared with diabetic or diabetic + insulin and their control.*

Conclusion: *Both ZnONPs and SNPs can be used to ameliorate the oxidative stress in the brain resulting from diabetes mellitus.*

Keywords: Oxidative stress, antioxidants, diabetic brain, nanoparticles.

INTRODUCTION

Diabetes mellitus refers to a group of metabolic impairments characterisation by high blood glucose levels (1). The greatest consequence of diabetes mellitus is the generation of reactive oxygen species (ROS) (2). The ROS can induce B-cell failure and develop insulin resistance (3). Diabetes mellitus can directly affect brain cells through the generation of oxidative stress, leading to apoptosis (4). The key mechanism is illustrated as an increase in glucose metabolism due to hyperglycaemia that promotes mitochondrial respiration, resulting in a release of superoxide and other reactive oxygen or nitrogen species into the cytoplasm (5).

Zinc has been reported to play a direct role in glucose homeostasis by enhancing hepatic glycogenesis through its actions on the insulin-signalling pathway. Thus, it improves glucose utilisation (6), inhibits intestinal glucose absorption (7) and increases glucose uptake in skeletal muscle and adipose tissue (6). Moreover, zinc is reported to inhibit glucagon secretion (8), thus reducing gluconeogenesis and glycogenolysis. It also enhances the structural integrity of insulin (9).

Decreased zinc in the pancreas may reduce the ability of the islet β-cells to synthesise and lead insulin into the blood (10). Furthermore, knowing zinc's antioxidant role (11), reduced zinc may exacerbate the oxidative stress-mediated complications of diabetes.

In recent years, there has been a great development of nanotechnology in the field of science and technology. Metallic nanoparticles, such as gold, silver, zinc, and metal oxide nanoparticles, have shown great challenges in the field of medicine (12).

In a previous study, we proved the antidiabetic effect of zinc oxide (ZnONPs) and silver (SNPs) nanoparticles

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as a novel agent to control diabetes mellitus in rats (13). The aim of this study is to test the ability of ZnONPs and SNPs to reduce the oxidative stress induced by diabetes mellitus in the brain tissues of diabetic rats.

MATERIALS AND METHODS

Animals

Fifty male albino rats, weighing 120 ± 20 g at the beginning of the experiment, were selected. All rats were placed into five groups. The first—the control (N = 10)—was not subjected to any treatment. The other groups included the remaining 40 animals which were induced to be diabetic through the injection of a single dose (100 mg/kg) of streptozotocin (STZ) intraperitoneally (Sigma-Aldrich, Catalog No S0130, Seelze, Germany). The diabetic rats were further divided into four groups: diabetic rats (N-10) served as a positive control with no treatment; diabetic + ZnONPs were administered a daily dose of ZnONPs per-Os (Sigma-Aldrich, Catalog No.721077, Seelze, Germany) of 10 mg/kg; diabetic + SNPs group were administered a daily dose of SNPs Per-Os (Sigma-Aldrich, Catalog No. 730793, Seelze, Germany) of 10 mg/kg; and diabetic + insulin group were injected with a subcutaneous dose of insulin (0.6 units/50 g) for 30 consecutive days (13).

Ethical statement

All experimental procedures were performed in agreement with the Saudi Arabian laws and university guidelines for experimental animal care and rights.

Sampling protocol

One gram of brain tissue was collected in liquid nitrogen, and then divided into different aliquots which were preserved at –80°C until they were used in biochemical and molecular biological investigations.

Biochemical assay

Brain Zn and Ag concentrations were analysed with an inductively coupled plasma–atomic emission spectroscopy, using an Ultima 2 apparatus (Horiba Jobin, Yvon, France).

Brain homogenate was used for determination of CAT, GPx and GRD activity using a kit (Cat. No. NWK-CAT01, NWK-GPX01 and NWK-GR01) purchased from Northwest Life Science Specialties (NWLSSTM), Vancouver, Canada. Superoxide dismutase (SOD) activity was determined by using Cayman SOD diagnostic kit (Cat. No. 706002, Cayman, USA). Malondialdehyde (MDA) was analysed by measuring the production of thiobarbituric acid-reactive substances (TBARS) using TBARS assay kit (Cat. No. 10009055, Cayman, USA). Total antioxidant capacity (TAC) was determined by using a kit supplied by Bio-diagnostic (Cat. No. TA 2512, Giza, Egypt).

Molecular analysis

Brain SOD, CAT, GPx and GRD gene expression were quantified using real-time polymerase chain reaction (PCR). Total RNA was isolated from tissue samples using the RNeasy Mini Kit Qiagen (Cat. No.74104), and 0.5 µg of total RNA was used for production of cDNA, using Qiagen Long Range 2 Step RT-PCR Kit (Cat. No.205920). Approximately 5 µL of total cDNA was mixed with 12.5 µL of 2x SYBR® Green PCR mix, with ROX from BioRad, and 10 pmol/µL of each forward and reverse primer for the measured genes. The housekeeping gene, β-actin, was used as a constitutive control for normalisation. Primers were designed using Primer3 software (The Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as per the published rat SOD, CAT, GPx, GRD and β-actin gene sequences (Table 1) of NCBI database; all primers were

Table 1: Primers oligonucleotide sequences of SOD, CAT, GPx, GRD and β-actin genes

Gene	Oligonucleotides sequences		Size (bp)	Gene ID
SOD	F	5′-ATGGGGACAATACACAAGGC-3'	225	Z21917.1
	R	5'-TCATCTTGTTTCTCGTGGAC-3′		
CAT	F	5′-GTCCGATTCTCCACAGTCGC-3′	272	AH004967.1
	R	5′-CGCTGAACAAGAAAGTAACCTG-3′		
GPx	F	5′-CACAGTCCACCGTGTATGCC-3′	292	S50336.1
	R	5′-AAGTTGGGCTCGAACCCACC-3′		
GRD	F	5′-CCATGTGGTTACTGCACTTCC-3′	171	NM_053906
	R	5′-GTTCCTTTCCTTCTCCTGAGC-3′		
β-actin	F	5′-TCACTATCGGCAATGTGCGG-3′	260	NM_007393
	R	GCTCAGGAGGAGCAATGATG-3′ 5′-		

SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase, GRD = glutathione reductase.

provided by Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Polymerase chain reaction (PCR) reactions were carried out in an AbiPrism 7300 (Applied Biosystems, Waltham, MA, USA). The RNA concentration in each sample was determined from the threshold cycle (Ct) values. The messenger RNA (mRNA) expression levels were calculated relative to β-actin gene mRNA levels using the $2^{-DD\,CT}$ method.

Statistical analysis

The obtained data were analysed using SPSS version 20 (IBM 1 New Orchard Road Armonk, NY, USA). Data were presented as a mean ± SD (N = 10). Student's *t*-test was used to calculate the differences between groups at $p < 0.05$.

RESULTS

A significant increase in the activities and mRNA expression levels of SOD, CAT, GPx and GRD were shown in rats treated with ZONPs and SNPs, as compared with the diabetic rats (Tables 2 and 3). Malondialdehyde levels were significantly decreased where there was a significant increase in the Zn, Ag and TAC in the brain of ZnONP- and SNP-treated rats when compared with the diabetic or diabetic + insulin groups and the control (Tables 2 and 4).

DISCUSSION

In the present study, we examined the ameliorative effect of both ZnONPs and SNPs on the oxidative stress generated in brain cells of the diabetic rats. Both Zn and Ag particles were increased significantly in brain tissues of diabetic rats subjected to treatment with ZnONPs and SNPs. Zinc levels tended to be 32 ± 3 and 21 ± 5 µg/g and 7.3 ± 1.5 and 13 ± 3 µg/g for silver, when compared with their control: 22 ± 4 µg/g for Zn and 8 ± 1 µg/g for silver (Table 2). Long circulation of nanoparticles leads to an increased chance of their passage to tissues, and hence higher cellular uptake (14). Once taken up in cells, particles encounter an increasingly acidic environment as they move from early to late endosomes and finally to lysosomes, resulting in their dissolution (15). Such phenomena may have contributed to the observed increases in tissue Zn and Ag levels in our study. Administration of ZnONPs and SNPs in diabetic rats resulted in the prevention of the decrease in the activity and mRNA expression levels of SOD, CAT, GRD and GPx.

Table 2: Biochemical investigations in experimental rats administered zinc oxide and silver nanoparticles

Parameters	Control	Diabetic	Diabetic + ZnONPs	Diabetic + SNP	Diabetic + insulin
Zn (µg/g)	22 ± 4	14 ± 2**	32 ± 3$^{*\#\#}$	21 ± 5$^{\#}$	22.5 ± 3$^{\#}$
Ag (µg/g)	8 ± 1	7 ± 1.3	7.3 ± 1.5	13 ± 3$^{*\#\#}$	7.5 ± 2
SOD (µmol/mg wt.w/min)	32 ± 2	8.2 ± 2***	25 ± 3$^{*\#\#\#}$	18.7 ± 1.5$^{*\#}$	10.3 ± 1**
CAT (µmol/H_2O_2 decomposed/ mg P/min)	900 ± 20	500 ± 15***	790 ± 10$^{*\#\#}$	710 ± 20$^{*\#}$	550 ± 23**
GPx (µmol/mg P/min)	130 ± 13	48.3 ± 7.6***	100 ± 10$^{*\#\#\#}$	90 ± 3$^{*\#\#}$	50 ± 4***
GRD (U/mg P)	6.5 ± 0.5	2.8 ± 0.3***	6.3 ± 0.3$^{\#\#\#}$	4.4 ± 0.4$^{*\#\#}$	3 ± 0.5***

wt.w = wet weight tissue, SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase, GRD = glutathione reductase, SNP = silver nanoparticle, ZnONP = zinc oxide nanoparticle.
Results are tabulated as means ± SD of 10 animals. Statistical analyses were performed using two-tail Student's *t*-test.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ comparing to control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ comparing to the diabetic rats.

Table 3: Messenger RNA expression profile (relative expression to β-actin) of antioxidant genes in diabetic rats administered zinc oxide and silver nanoparticles

Genes	Control	Diabetic	Diabetic + ZnONPs	Diabetic + SNP	Diabetic + insulin
SOD	1.1 ± 0.001	0.2 ± 0.003**	0.9 ± 0.0 1$^{*\#\#}$	0.7 ± 0.002$^{*\#}$	0.7 ± 0.09$^{*\#}$
CAT	1.3 ± 0.002	0.3 ± 0.001***	1 ± 0.02$^{*\#\#}$	0.8 ± 0.01$^{*\#}$	0.6 ± 0.01$^{*\#}$
GPx	0.96 ± 0.06	0.44 ± 0.0 3**	0.9 ± 0.04$^{\#\#}$	0.72 ± 0.0 2$^{*\#\#}$	0.6 ± 0.05$^{*\#}$
GRD	0.9 ± 0.05	0.5 ± 0.002**	1.7 ± 0.3$^{*\#\#\#}$	1 ± 0.04$^{\#\#}$	0.7 ± 0.03$^{*\#}$

SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase, GRD = glutathione reductase, SNP = silver nanoparticle, ZnONP = zinc oxide nanoparticle.
Results are tabulated as mean ± SD of 10 animals. Statistical analyses were performed using two-tail Student's *t*-test.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ comparing to control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ comparing to the diabetic rats.

Table 4: Biochemical effect of zinc oxide, silver nanoparticles on malondialdehyde and total antioxidant capacity in brain tissue of diabetic rats

Parameters	Control	Diabetic	Diabetic + ZnONPs	Diabetic + SNP	Diabetic + insulin
TAC (μmg^{-1} wt.w)	6.1 ± 0.66	2.8 ± 0.3***	6.6 ± 0.6###	5.7 ± 0.6##	4 ± 0.9*#
MDA ($nmolg^{-1}$ wt.w)	0.95 ± 0.1	4.3 ± 0.3***	1.6 ± 0.3###	1.1 ± 0.4##	2.5 ± 0.05*#

MDA= malondialdehyde TAC= total antioxidant capacity, SNP = silver nanoparticle, ZnONP = zinc oxide nanoparticle. Results are tabulated as mean ± SD of 10 animals. Statistical analyses were performed using two-tail Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ comparing to control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ comparing to the diabetic rats.

We attributed the high activities and expression levels of antioxidant enzymes in the brains of ZnONP- and SNPs-administered rats to the effect of these nanoparticles improving the insulin secretion and amelioration of the oxidative stress induced due to impairment of glucose homeostasis (13). These enzymes play a pivotal role in the elimination of free radicals from the tissues (16), so their high activities and expression levels in brain cells protect them from the effects of oxidative stress.

Diabetes mellitus is always associated with the generation of ROS (17), and this is clear in our study proved by a reduction in both activities and expression of the examined antioxidant enzymes in diabetic, untreated rats. In the same line of our study, the over production of superoxide free radicals is eliminated by the overexpression of antioxidant enzymes (18). Malondialdehyde was used as a potent marker for oxidative stress (19). Many authors have cited that diabetes mellitus is usually associated with an increase in MDA production and therefore increased their levels in tissues and blood in diabetic models (20). Our results showed a significant increase in MDA levels in the brains of diabetic rats. We attribute this increase to the oxidative stress generated due to high glucose levels as mentioned before. In ZnONPs and SNPs, the levels of MDA were decreased in the brain tissues. The reduction of MDA levels in the brain of nanoparticle-administered rats may be due to the activity of these nanoparticles to improve the insulin secretion (13) and the increase in the SOD, CAT, GRD and GPx activities and mRNA expression. To demonstrate the TAC of the brain in diabetics and diabetic rats administered ZnONPs and SNPs, the TAC was measured in the brain of all rats. The highest capacity was observed in diabetic rats administered ZnONPs, followed by diabetic rats administered SNPs, when compared to diabetic rats and control (Table 4). We attribute the high TAC in nanoparticle-administered rats to the ability of these particles to improve the antioxidant power of the cells proved recently by Kunjiappan *et al.* (21).

In all examined rats, the effect of ZnNPs and SNPs was more obvious than the insulin in diabetic rats. There is more improvement in total antioxidant power in the brain of diabetic rats administered nanoparticles than in those treated with insulin. Up till now, we have not had a clear explanation about the action of these nanoparticles. But the speed at which these nanoparticles were up taken by the cells (14, 15), and its ability to induce endogenous insulin secretion (13), may be the usual cause of their ability to improve the antioxidant capacity in the brain cells.

CONCLUSION

Both ZnONPs and SNPs are potent agents for improvement of the antioxidant power of brain cells. They have the ability to protect brain cells from the oxidative stress generated in diabetes mellitus.

AUTHORS' NOTE

The authors declare that they have no conflict of interest.

REFERENCES

1. Lin Y, Sun Z. Current views on type 2 diabetes. J Endocrinol 2010; **204:** 1–11.
2. Harrison D, Griendling KK, Landmesser U,Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. Am J Cardiol 2003; **91:** 7–11.
3. Stephens JW, Khanolkar MP, Bain SC. The biological relevance and measurement of plasma markers of oxidative stress in diabetes and cardiovascular disease. Atherosclerosis 2009; **202:** 321–9.
4. May JM, Jayagopal A, Qu ZC, Parker WH. Ascorbic acid prevents high glucose-induced apoptosis in human brain pericytes. Biochem Biophys Res Commun 2014; **452:** 112–7.
5. Baynes JW. Role of oxidative stress in development of complications of diabetes. Diabetes 1991; **40:** 405–12.
6. Jansen J, Karges W, Rink L. Zinc and diabetes—clinical links and molecular mechanisms. J Nutr Biochem 2009; **20:** 399–417.
7. Ueda E, Yoshikawa Y, Sakurai H, Kojima Y, Kajiwara NM. In vitro alpha-glucosidase inhibitory effect of Zn (II) complex with 6-methyl-2-picolinmethylamide. Chem Pharm Bull 2005; **53:** 451–2.
8. Egefjord L, Petersen AB, Bak AM, Rungby J. Zinc, alpha cells and glucagon secretion. Curr Diabetes Rev 2010; **6:** 52–7.
9. Sun Q, Van Dam RM, Willett WC, Hu FB. Prospective study of zinc intake and risk of type 2 diabetes in women. Diabetes Care 2009; **32:** 629–34.
10. Meyer JA, Spence DM. A perspective on the role of metals in diabetes: past findings and possible future directions. Metallomics 2009; **1:** 32–41.
11. Chausmer AB. Zinc, insulin and diabetes. J Am Coll Nutr 1998; **17:** 109–15.

12. Hirst SM, Karakoti A, Singh S, Self W, Tyler R, Seal S et al. Bio-distribution and in vivo antioxidant effects of cerium oxide nanoparticles in mice. Environ Toxicol 2013; **28:** 107–18.
13. Alkaladi A, Abdelazim A, Afifi M. Antidiabetic activity of zinc oxide and silver nanoparticles on streptozotocin-induced diabetic rats. Int J Mol Sci 2014; **15:** 2015–23.
14. Li SD, Huang L. Pharmacokinetics and bio distribution of nanoparticles. Mol Pharm 2008; **5:** 496–504.
15. Nel AE, Mädler L, Velegol D, Xia T, Hoek EM, Somasundaran P et al. Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater 2009; **8:** 543–57.
16. Tanaka M, Mokhtari GK, Terry RD, Balsam LB, Lee KH, Kofidis T et al. Overexpression of human copper/zinc superoxide dismutase (SOD1) suppresses ischemia-reperfusion injury and subsequent development of graft coronary artery disease in murine cardiac grafts. Circulation 2004; **14:** 110.
17. Telci A, Cakatay U, Kayali R. Oxidative protein damage in plasma of type 2 diabetic patients. Horm Metab Res 2000; **32:** 40–3.
18. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000; **404:** 787−90.
19. Tappel AL. Lipid peroxidation damage to cell components. Clin Pathol Fed Proc 1973; **32:** 1870–4.
20. Baş H, Kalender Y, Pandir D, Kalender S. Effects of lead nitrate and sodium selenite on DNA damage and oxidative stress in diabetic and non-diabetic rat erythrocytes and leucocytes. Environ Toxicol Pharmacol 2015; **39:** 1019–26.
21. Kunjiappan S, Bhattacharjee C, Chowdhury R. In vitro antioxidant and hepatoprotective potential of Azolla microphylla phytochemically synthesized gold nanoparticles on acetaminophen-induced hepatocyte damage in Cyprinus carpio L. In Vitro Cell Dev Biol Anim. 2015; **51:** 630–43.

CASE REPORT

49, XXXXY: The Role of MSX1 Gene

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ABSTRACT

49, XXXXY syndrome is the most severe and rare form of Klinefelter syndrome. The number of cases of oral and dental findings with this syndrome is very little. The aims of our case report are to present oral and dental findings of the syndrome about which we have limited knowledge, to support other case reports, and to evaluate the relationship between this syndrome in which hypodontia is seen and the MSX1 *gene that is associated with hypodontia. A clinical and radiographic examination was performed on an 11-year-old patient who had been taken to a practitioner at age 1 for delayed development, diagnosed with 49, XXXXY syndrome by chromosome analysis, and exhibited characteristic features of the syndrome. Congenital missing permanent teeth, hypertaurodont primary and permanent teeth, delayed tooth development and mandibular prognathism were observed in the examination. Whether there were any mutations in the* MSX1 *gene was researched, and the association with this syndrome was evaluated.*

Keywords: Hypodontia, Klinefelter syndrome, *MSX1*, taurodontism, XXXY syndrome.

INTRODUCTION

Klinefelter syndrome is defined as male hypogonadism arising from two or more X chromosomes and one or more Y chromosomes (1). It is the most common chromosomal anomaly and its most severe and rarely recorded constitution is 49, XXXXY (2). In this type of syndrome, of which prevalence is approximately 1 in 85,000 newborn males, there are very few cases available where oral and dental findings have been reported (3, 4).

Symptoms of the syndrome have been reported to include mental retardation, cardiac anomalies, hypogonadism, small penis, abnormal scrotum, cryptorchism, limited elbow flexion/extension, radioulnar synarthrosis, clinodactyly, coxa valga, genua valga, gap between first and second toes, flat feet, age-related decline in bone, hardened cranial sutures, defect in capitate bone, thoracic kyphosis, and scoliosis. Craniofacial features of the syndrome include ocular hypertelorism, upslanting palpebral fissures, epicanthic folds, diplopia, a broad flat nose, malformed ears, and mandibular prognathism (5). Among the oral and dental findings reported, there are bifid uvula or cleft palate (4, 6), congenitally missing permanent teeth (7, 8), taurodontism (8–10), spade-shaped cutting teeth (9), and dentine defects (6).

In recent years, the number of dental studies which have made use of genetics have increased and both patients and physicians have been provided with more information regarding these diseases. However, although numerous authors have reported distinctive features of this syndrome, genetic studies related to the syndrome are limited. Differences in the clinical manifestations of the syndrome with regard to age are associated with changes in hormonal levels. Redundancy of X chromosomes leads to androgen deficiency. Loss of feedback inhibition of the pituitary gland depending on this deficiency results in elevated levels of gonadotropin (11). Taurodontism, also included among dental findings, is associated with the redundancy of X chromosomes (12, 13). The most studied and emphasised gene responsible for missing teeth, which is also counted among the dental findings of this syndrome, is *MSX1*. *MSX1* is a transcription factor closely associated with the genetic network regulating dental development. Mutations

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revealed in *MSX1*, which is synthesised during the development and migration of neural crest, cause missing teeth.

Our case report is aimed to present oral and dental findings on this syndrome, for which we have limited knowledge. Several cases of Klinefelter syndrome accompanied by missing teeth are reported, but no relationships of those with *MSX1* have been presented. The role of the *MSX1* gene, which is associated with missing teeth in this syndrome, will be evaluated as well.

CASE REPORT

Anamnesis of an 11-year-old patient named K.K., who was brought into our clinic due to toothache and widespread dental caries, revealed that the patient had seen a physician because of the complaint of growth retardation at one year of age and had been diagnosed with 49, XXXXY syndrome *via* chromosomal analysis. The parents, who stated that they are not relatives although they lived in the same village, reported that no similar findings were observed in their other two children, who were older than K.K., or among their relatives. The mother stated that she received an iron-containing medicine during her pregnancy, that she delivered the baby normally by induced labour two days after her due date, and that the baby weighed 2500 g. It was learned that the patient, with mental retardation of 75%–80%, started to speak and walk at five years of age.

In the general examination, ocular hypertelorism, upslanting palpebral fissures, epicanthal folds, diplopia, a broad, flat nose (Fig. 1A), limited elbow flexion/extension, kyphosis (Fig. 1B), a small penis, abnormal scrotum, and palmar, and plantar hyperkeratotic areas were recorded.

In the oral examination, prognathic mandibula, poor oral hygiene and widespread dental caries (Fig. 2) were detected.

In the radiographic examination, it was observed that he lacked teeth 18, 15, 14, 12, 22, 25, 28, 38, 35, 34, 44, 45, 48, and had hyper and mesotaurodont deciduous and permanent teeth (Fig. 3). Using the Haavikko method, the dental age of the patient was determined to be 1.64 years less than the chronological age. The patient was within the pp2 balance period in terms of skeletal development, according to hand-wrist X-rays. When his hand-wrist radiography was assessed, he was found to be seven years of age.

After informing the patient and the parents about the procedure of blood sample drawing, the planned

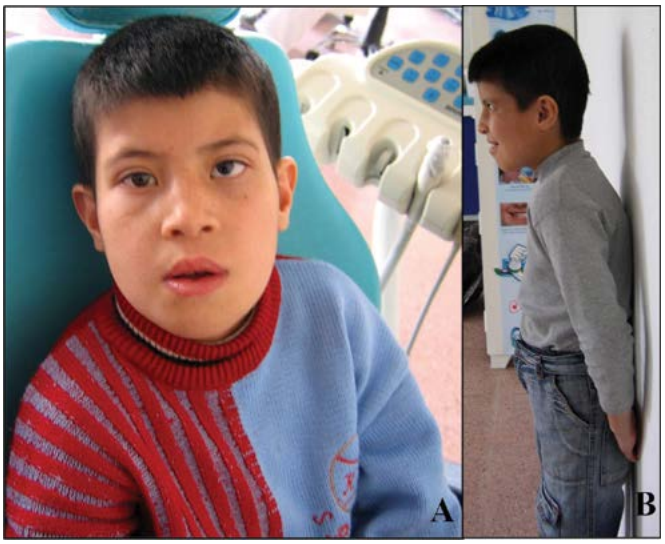

Fig. 1: Patient with Klinefelter's syndrome has ocular hypertelorism, upslanting palpebral fissures, epicanthal folds, a broad flat nose (A) and kyphosis (B).

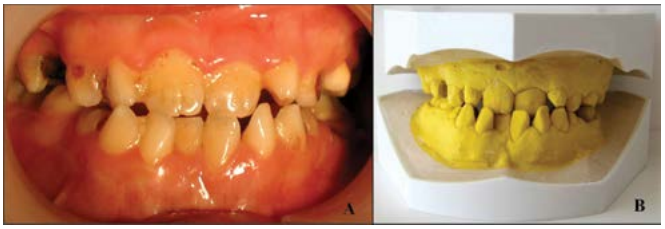

Fig. 2: Intraoral view of the patient before dental treatment (A) and the appearance of the stone model (B).

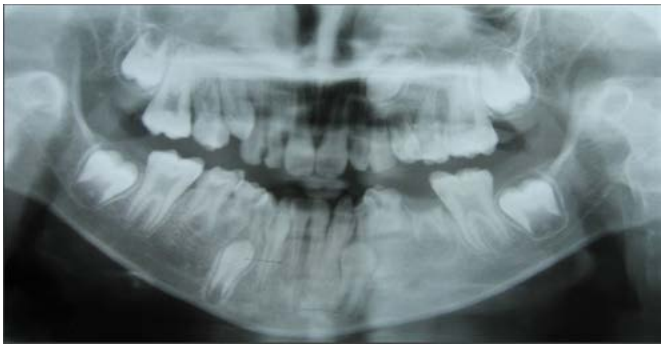

Fig. 3: The radiographic appearance of the patient before dental treatment.

treatment to be performed, and possible problems, their written consent was obtained.

A prevention programme was created because the patient had to be fed frequently with a soft diet, was incapable of maintaining oral hygiene, came from a family with low socio-economic status, and experienced problems with cooperation. The patient was trained by his parents in teeth brushing and received dietary recommendations. No complete cooperation could be established with the patient. However, composite

restoration of permanent, maxillary, left, central incisor; compomer restoration of primary maxillary and mandibular left canines, and metal-reinforced, glass, ionomer-cement restoration of permanent first molars; permanent, maxillary, left, first premolar; primary, mandibular first molars; primary, mandibular, right, second molar; and primary, mandibular, left, canine were performed. Primary maxillary second molars and primary mandibular left second molar have deep caries and excessive loss of substance, and primary mandibular lateral incisors present within the mouth were scheduled to be extracted (Fig. 4). Following the necessary treatments and extraction procedures, a follow-up programme was created for the patient to receive fluorine to maintain oral hygiene, to check tooth restorations, and to monitor the period of growth and development.

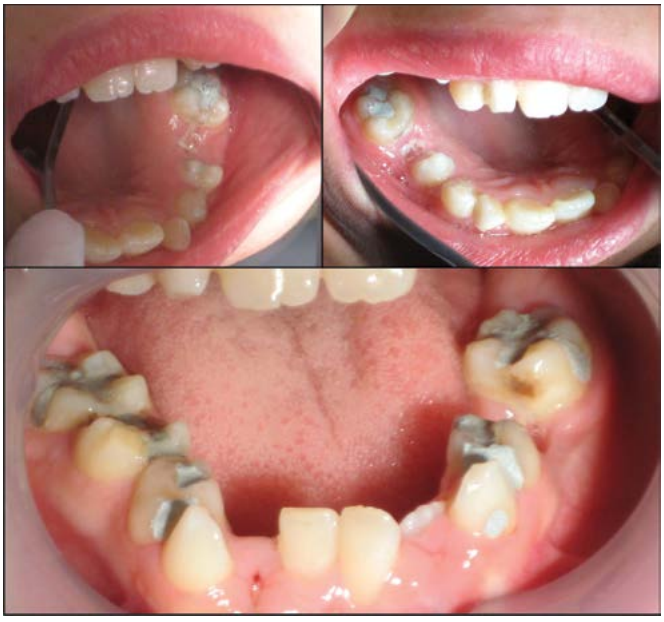

Fig. 4: Intraoral view of the patient after dental treatment.

Written, informed consent was obtained from the patient to take and use the blood and DNA samples for diagnostic purpose. Genomic DNA was extracted from whole blood samples for molecular analyses by using DNA isolation kit (Gentra, Qiagen, Valencia, CA, USA). Amplification of polymerase chain reaction (PCR) was performed using overlapping primer sequences (http://genetics.uiowa.edu/publications/peterj/PeterMSX190902.html), which were designed to include two coding exons of the *MSX1* gene and exon–intron binding sites. These sequences consisted of all coding sequences of exon1 and exon2, 468 nucleotides beginning from 5′ end of the start codon (whole 5′ UTR), and 1073 nucleotides after the stop codon (whole 3′ UTR).

The volume of 25 µl was used for PCR reaction, and 50-ng DNA, 0.2-mM dNTP, 2.5-mM $MgCl_2$, and 10-pmol and 1U Taq DNA polymerase enzymes for each primer sequence were applied. Polymerase chain reaction conditions were carried out as 40 seconds at 95°C, 35 seconds at 58°C, 40 seconds and 35 cycles at 72°C, and the final elongation stage was performed at 72°C for 5 seconds. Polymerase chain reaction products obtained were exposed to direct DNA sequence analysis by ABI 3100 DNA sequencer (Applied Biosystems, Thermo Fisher, Foster City, CA, USA). As a result of these analyses, it was determined that the second exon of the patient was subjected to complete deletion. The deletion occurred in a sequence including a total of 696 bp [del 4 864 207–4 864 902] (Fig. 5). It covered a part of the intronic region between exons, which consisted of 221 bp and was close to the second exon; the whole coding sequence of the second exon with 443 bp, and the following sequence of 42 bp belonging to 3′ UTR.

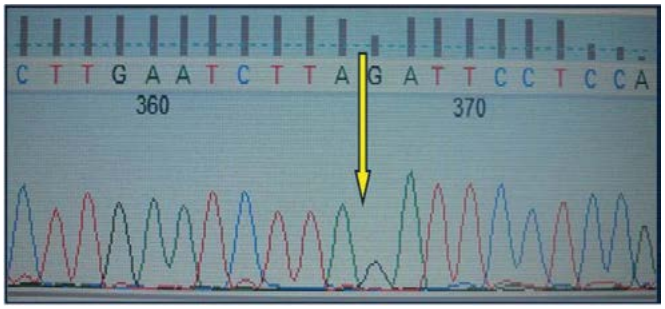

Fig. 5: DNA sequence chromatogram in forward direction showing the 696 bp deletion (de l4 864 207 – 4 864 902) of *MSX1* gene that encompasses the whole of the second exon. The arrow mark indicates the range of deletions that occurred. While the series at the left side of the arrow are belonging to the intronic region, and the series at the right side are belonging to the 3' UTR region.

RESULT AND DISCUSSION

Among the oral and dental findings of the syndrome, no bifid uvula, cleft palate, spade-shaped, cutting teeth and dentine defects were recorded in our patient. Prognathic mandible, missing teeth and findings of taurodontism were included among the symptoms which might be observed related to this syndrome. Depending on the mutations of the *MSX1* gene, the most frequently recorded missing teeth are the permanent second premolars and the permanent third molars (14). More rarely, losses of permanent, maxillary, first premolars; permanent, mandibular, first molars; permanent, maxillary, lateral incisors; and permanent, mandibular, central incisors might be seen. Deciduous teeth development was normal, in general (15). Consistent with these findings,

teeth 18, 15, 14, 12, 22, 25, 28, 38, 35, 34, 44, 45, 48 were determined to be missing in our patient. Deciduous and permanent molar teeth of the patient exhibited hyper- and mesotaurodontism. Deeply localised pulpal floor in taurodontic teeth and the complex structure of the root canal system complicate the use of canal tolls, the determination of canal localisation and the filling of canals; thus, an indication for teeth extraction is usually established. Missing teeth reveal aesthetic and psychological problems by affecting teeth development and mandibular bone and cause a negative effect on the growth-development period of individuals.

MSX homeobox genes are expressed in epithelial or mesenchymal cells during morphogenesis. *MSX1* and *MSX2* genes in vertebrates are expressed in embryonic tissues, such as mandibular and dental tissues, which show epithelial and mesenchymal interaction during morphogenesis. The *MSX1* gene, localised between 4 861 406–4 865 663 nucleotides on the fourth chromosome, codes a protein including 297 amino acids.

As a result of the mutation which occurred, 156 amino acids, coded only by the first exon, were found as normal, but the remaining 141 amino acids could not be coded. While amino acids of protein, included between 166 and 225, constitute a DNA binding site, this region completely disappeared due to mutation; thus, there are no DNA binding sites in the protein.

MSX1 protein is responsible for the repression of gene transcription by interacting with TATA-box binding protein. A new 'premature *MSX1* protein' revealed a structural change in the gene which is considered to especially affect the craniofacial development and odontogenesis.

We believe that this new protein could not perform its function due to the complete disappearance of DNA of the binding site, and it generates the primary reason for missing teeth recorded in the patient. Moreover, we think that other clinical findings observed in the patient may also have arisen from the premature protein because of the fact that *MSX1* is a transcription factor effective in the early development of neural crest.

REFERENCES

1. Kumar V, Abbas AK, Fausto N. Robbins and Cotran pathologic basis of disease. Philadelphia: Elsevier Saunders; 2005.
2. Nielsen J, Pelsen B, Sorensen K. Follow-up of 30 Klinefelter males treated with testosterone. Clin Genet 1988; **33:** 262–9.
3. Pallister PD. 49, XXXXY syndrome. Am J Med Genet 1982; **13:** 337–9.
4. Linden MG, Bender BG, Robinson A. Sex chromosome tetrasomy and pentasomy. Pediatrics 1995; **96:** 672–82.
5. Gorlin RJ, Cohen MM, Hennekam RCM. Syndromes of the head and Neck. USA: Oxford University Press; 2001.
6. Zaleski WA, Houston CS, Pozsonyi J, Ying KL. The XXXXY chromosome anomaly: report of three new cases and reviews of 30 cases from literature. Can Med Assoc J 1966; **94:** 1143–54.
7. Sheriden MK, Radlinski S. Brief report: a case study of an adolescent male with XXXXY Klinefelter's Syndrome. J Autism Dev Disord 1988; **18:** 449–56.
8. Hata S, Maruyama Y, Fujita Y, Mayanagi H. The dentofacial manifestation of XXXXY syndrome: a case report. Int J Paediatr Dent 2001; **11:** 138–42.
9. Gardner DG, Girgis SS. Taurodontism, shovel-shaped incisors and the Klinefelter syndrome. Dent J 1978; **44:** 372–3.
10. Jasper MT, Witkop CJ. Taurodontism, an isolated trait associated with syndromes and X-chromosomal aneuploidy. Am J Hum Genet 1980; **32:** 396–413.
11. Joseph M. Endodontic treatment in three taurodontic teeth associated with 48,XXXY Klinefelter syndrome: a review and case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008; **105:** 670–7.
12. Keeler C. Taurodont molars and shovel shaped incisors in Klinefelter's syndrome. J Hered 1973; **64:** 234–6.
13. Varella J, Alvesalo L. Taurodontism in females with extra X chromosomes. J Craniofac Genet Dev Biol 1989; **9:** 129–33.
14. Nieminen P. Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol 2009; **312B:** 320–42.
15. Forestier IB, Molla M, Verloes A, Berdal A. The genetic basis of inherited anomalies of the teeth: Part 1: Clinical and molecular aspects of non-syndromic dental disorders. Eur J Hum Genet 2008; **51:** 273–91.

CASE REPORT

Foetus in Foetu: A Rare Prenatal Diagnosis of a Foetal Abdominal Mass

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ABSTRACT

A pregnant woman was referred to our perinatal tertiary care centre because of foetal bowel distension. Ultrasonography revealed a well-circumscribed circular mass in the foetus' abdomen. After delivery, the male neonate underwent laparotomy to excise the mass. Pathologic examination of the mass was consistent with the diagnosis of foetus in foetu. This case of foetus in foetu was differentiated from a teratoma based on the presence of a vertebral axis and appropriate arrangement of other organs and limbs relative to the vertebral axis. A multidisciplinary clinical approach may be more suitable for managing rare foetal disorders, such as this one.

Keywords: Abnormal twinning, enclosed twin, foetus in foetu, intra-abdominal foetal mass.

INTRODUCTION

Most neonatal abdominal masses are caused by benign retroperitoneal lesions such as hydronephrosis and multicystic dysplastic kidney (1). Foetus in foetu (FiF), also known as an enclosed twin, is a rare condition. It is described as a twin that 'fails to thrive' and is confined in its sibling. It is estimated to affect 1 in 500 000 live births (2). Isaacs reported that FiF was responsible for 25/534 (4.68%) of perinatal germ cell tumours (3). The first case was reported by Young in 1809 (4). Foetus in foetu is distinguished from a teratoma by the presence of a vertebral axis and, commonly, by the appropriate arrangement of other organs and limbs relative to the vertebral axis (5). In 80% of cases, the FiF was found in the retroperitoneum, while other, atypical sites include: the intracranial area (6, 7), mediastinum, lung, oropharynx, scrotum, adrenal gland, ovary, or the neck (8). Because of its malignant potential, the FiF should be completely excised by surgery. The prognosis after resection is usually benign (9).

CASE REPORT

A 31-year-old multiparous pregnant woman was referred to our perinatal tertiary care centre after a routine ultrasound scan showed that her 37-week-old foetus had bowel distension. The woman was in a non-consanguineous marriage and had no significant medical or gestational history. There was no history of twin gestation. A detailed sonogram and a follow-up ultrasound of the index pregnancy revealed no notable clinical signs.

A senior obstetrician examined the patient via ultrasound and noted that the foetal biometry was consistent with a gestational age of 34–35 weeks, there was severe oligohydramnios. There was a well-circumscribed circular mass measuring 44 × 38 × 40 mm with echoes consistent with calcification. The mass was located in the central upper abdomen, below the liver, in front of and between two foetal renal pelvises (Fig. 1A).

The male neonate was delivered via Caesarean section at 37 weeks and 1 day of gestation, because of foetal distress. The birth weight was 2740 g. The Apgar scores were 7 and 9 at 1 and 5 minutes, respectively. The mother was discharged 2 days after delivery, with no remarkable medical concerns at this time.

A physical examination of the neonate revealed that the only notable sign was mild to moderate abdominal distension. The neonate's complete blood count and arterial blood gas were normal. Ultrasound confirmed

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that the locations and structures of the liver, gall bladder, spleen and suprarenal glands were normal. The mass in the neonate's abdomen had the same features as those observed in the prenatal examination. Respiration and alimentation were noted as being normal. An experienced paediatric cardiologist reported that the neonate's cardiac system was normal. A computed tomography scan confirmed the ultrasound and physical examination findings (Fig. 1B).

The paediatric surgical team decided to perform a laparotomy to excise the mass. In open view during surgery, the mass compressed the duodenum from the posterior side (Fig. 1C). After opening the retroperitoneal space, it was possible to see the haemorrhagic structure of the mass (Fig. 1D). Excision of the mass eliminated the compression on the duodenum (Fig. 1E).

The mass was removed intact and sent for pathological and genetic examination. The mass weighed 220 g and measured 40 × 30 × 25 mm. A radiograph of the

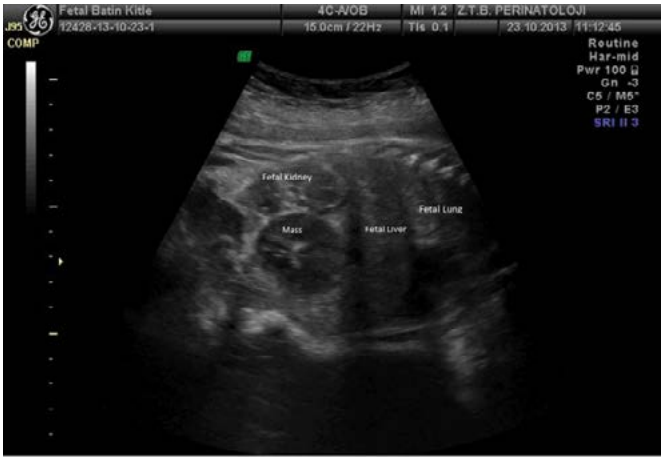

Fig. 1A. Coronal plane ultrasound view of the mass which located in the foetal abdomen.

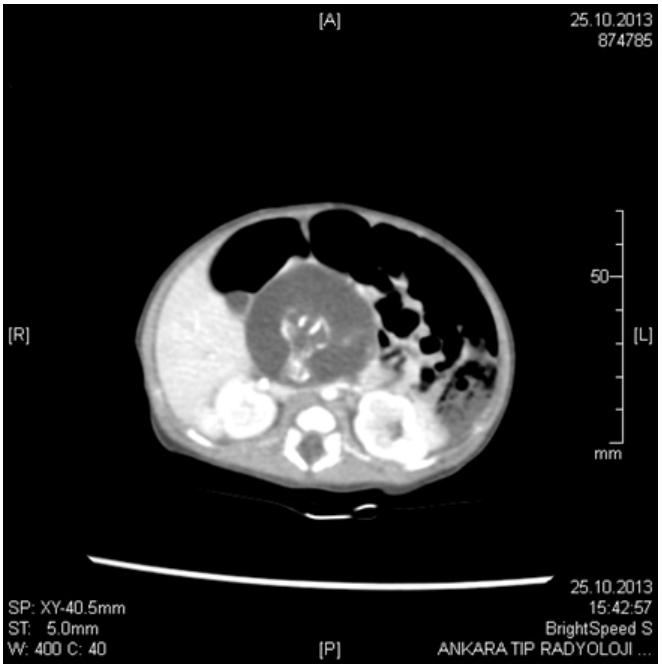

Fig. 1B. Computed tomographic view of the neonate shows the mass in the central abdomen. This view also shows the kidneys, liver and mildly dilated bowel of the neonate.

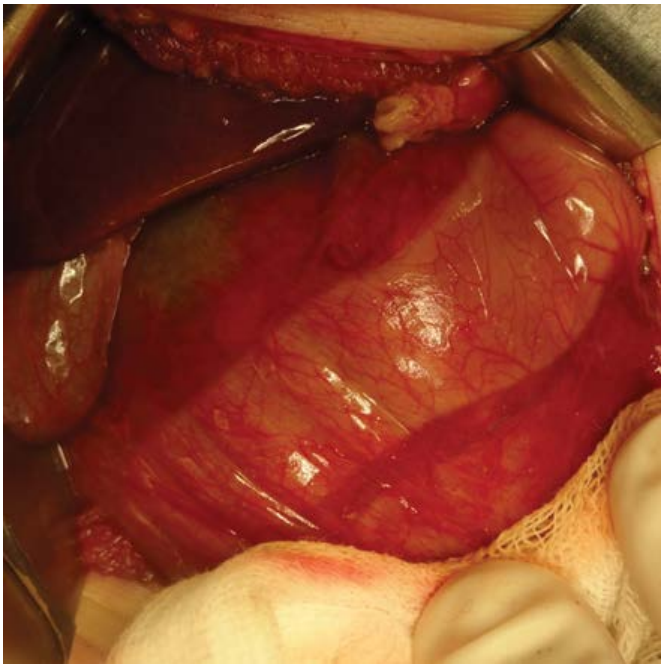

Fig. 1C. An open view of the neonate's liver, gall bladder and duodenum. The mass compresses the duodenum from the posterior side.

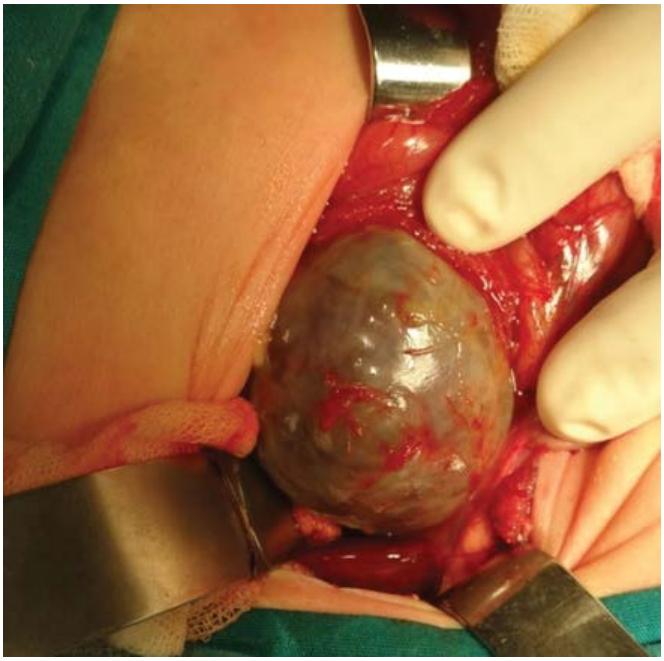

Fig. 1D. Image of the mass in the retroperitoneal space showing its congestive and haemorrhagic structure.

mass showed a linear calcification and the presence of a vertebral column and limbs (Fig. 1F). Pathologic and macroscopic examination of the mass revealed its haemorrhagic structure, including the presence of fatty tissue, irregularly arranged bone, cartilage, and a skin-covered conglomeration of recognisable rudimentary foetal parts (Fig. 1G). In addition, a rudimentary axial skeleton was found with three rudimentary limbs; the largest limb was 15 mm long and the smallest limb was 10 mm long. Microscopic examination confirmed that the mass was covered with skin and contained some osseous tissue and a rudimentary gastrointestinal tract. The final pathologic report stated that the mass was consistent with an acephalic acardiac foetus. Cytogenetic examination of the mass revealed that the karyotyping was 46 XY.

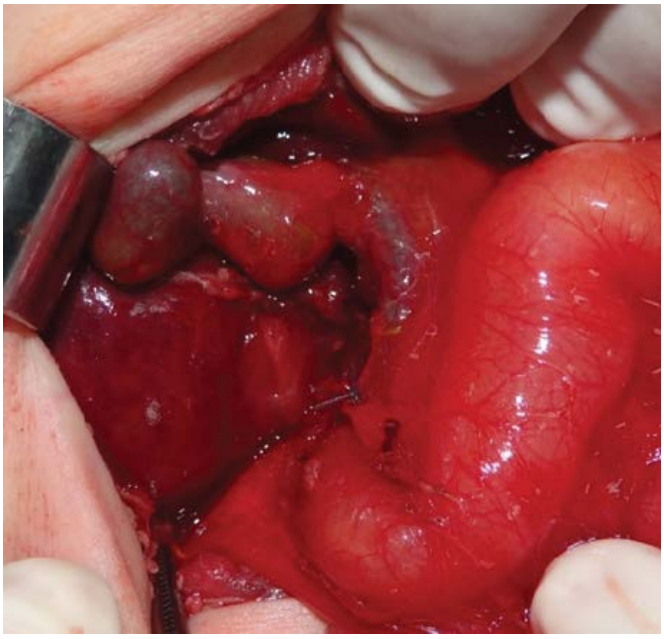

Fig. 1E. Excision of the mass eliminated the compression on the duodenum.

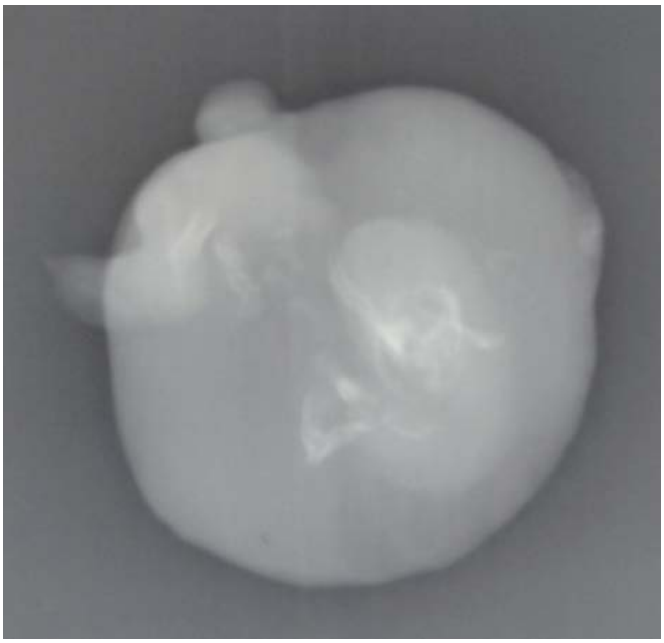

Fig. 1F. X-ray image of the mass.

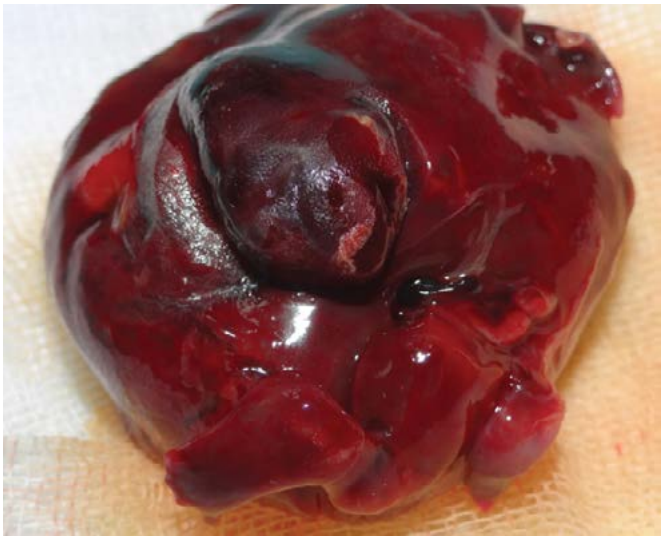

Fig. 1G. Internal view of the mass after removing its outer membrane. Structures consistent with limbs, a face and a gastrointestinal tract can be seen.

The neonate has experienced no complications at the last follow-up, 6 months after surgery.

DISCUSSION

This case of FiF was differentiated from a teratoma by the presence of a vertebral axis and the appropriate arrangement of other organs and limbs relative to the vertebral axis (5). The FiF also had a similar appearance to other cases reported in the literature.

Most authors have attributed the etiology of FiF to anomalous cleavage of monozygotic twins, where one foetus fails to thrive and is entrapped by the other foetus when the anterior abdominal wall closes around the umbilicus and linea alba (10). Therefore, the FiF must be entrapped during the second half of the first trimester or in the early phase of the second trimester.

Although FiF is more common in females (11), the gender of our neonate was male. The FiF was localised in the retroperitoneal space, the most commonly reported site.

Prenatal diagnosis of this rare condition may be difficult because of other possible diagnoses, including hydronephrosis, cystic abnormalities, solid tumours of the kidney, intestinal malformations, teratoma, choledochal cyst, neuroblastoma, adrenal haemorrhage, and infra-diaphragmatic pulmonary sequestration (12).

Ultrasound in the second trimester did not reveal a possible intra-abdominal mass in the present case, as in an earlier case reported by Huddle *et al*. (7). Therefore, FiF might not be visible until late pregnancy.

Most clinicians agree on the superiority of ultrasound imaging in everyday clinical practice, but it may be unsatisfactory in some circumstances. In the management of this case, it is possible that antepartum magnetic resonance imaging could have aided the diagnosis of FiF antenatally, or allowed us to exclude other types of intra-abdominal foetal mass from the differential diagnosis, if it had been performed. Nevertheless, pathologic examination is essential to reach the final diagnosis in such cases because all screening and imaging methods have some limitations that could hinder accurate diagnosis.

Huddle *et al*. highlighted the need for further genetic investigations, such as microarray or single nucleotide polymorphism analyses (7). It may be valuable to use these tools beyond conventional cytogenetic analysis because they can provide additional information regarding the identity of twins. Nevertheless, we think that the decision to use these tools must be clinically justified.

In conclusion, FiF is a rare clinical condition and is seldom considered in the differential diagnosis of a foetal intra-abdominal mass. If a clinician suspects a foetal abdominal mass, he/she should contact to an expert perinatologists and paediatric surgeons. Wherever possible, the foetus should be delivered in a clinic with suitable facilities, including an expert obstetric team, paediatric surgery, and neonatal intensive care unit. A multidisciplinary clinical approach may be more suitable for the management of such rare cases.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1. Hartman GE, Shochat SJ. Abdominal mass lesions in the newborn: diagnosis and treatment. Clin Perinatol 1989; **16:** 123–35.
2. Grant P, Pearn JH. 'Foetus-in-foetu'. Med J Aust 1969; **1:** 1016–9.
3. Isaacs H Jr. Perinatal (fetal and neonatal) germ cell tumors. J Pediatr Surg 2004; **39:** 1003–13.
4. Young GW. Case of a foetus found in the abdomen of a boy. Med Chir Trans 1809; **1:** 234–62.
5. Willis RA. The borderline of embryology and pathology. In: The embryonic tumours and teratomas. London, UK: Butterworth and Co., 1958: 147.
6. Goldstein I, Jakobi P, Groisman G, Itskovitz-Eldor J. Intracranial fetus-in-fetu. Am J Obstet Gynecol 1996; **175:** 1389–90.
7. Huddle LN, Fuller C, Powell T, Hiemenga JA, Yan J, Deuell B et al. Intraventricular twin fetuses in fetu. Neurosurg Pediatr 2012; **9:** 17–23.
8. Tofigh AM, Kavyani A, Abdollahi SM, Kazemzadeh G, Salman DN. Fetus in fetu: report of a case and literature review. Int J Surg 2008; **6:** e94–e96.
9. Khadaroo RG, Evans MG, Honore LH, Bhargava R, Phillipos E. Fetus in fetu presenting as cystic meconium peritonitis: diagnosis, pathology, and surgical management. J Pediatr Surg 2000; **35:** 721–3.
10. Hoeffel C, Khoang Q, Tran T, Fornes P. Fetus in fetu: a case report and literature review. Pediatrics 2000; **105:** 1335–44.
11. Spencer R. Parasitic conjoined twins: external and internal (fetus in fetu and teratomas), and detached (acardiacs). Clin Anat 2001; 14: 428–44.
12. Chandler JC, Gauderer MWL. The neonate with an abdominal mass. Pediatr Clin N Am 2004; **51:** 979–97.

CASE REPORT

Laryngocele Causing Airway Obstruction: A Unique Case Report

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ABSTRACT

Laryngocele is an abnormal cystic dilatation of the saccule or appendix of the laryngeal ventricle filled with air and connected to the lumen of the larynx. They rarely cause clinical complaints. However, still being rare clinical occasions, they may cause airway obstruction which require emergency interventions. In this report, we presented an extremely rare case of laryngocele which causes airway obstruction. A 35-year-old male was admitted due to hoarseness, progressive respiratory distress and swelling at the right side of the neck for three years. Laryngoceles are rarely symptomatic but because of their potential to cause sudden airway obstruction, they deserve serious attention.

Keywords: Airway obstruction, laryngocele, neck mass

INTRODUCTION

Laryngocele is an abnormal cystic dilatation of the saccule or appendix of the laryngeal ventricle filled with air and connected to the lumen of the larynx (1). Three types of laryngoceles have been described as internal, external and mixed types, in relation to the position of the sac with respect to the thyrohyoid membrane (2). Laryngoceles are usually asymptomatic. They rarely cause clinical complaints. However, still being rare clinical occasions, they may cause airway obstruction which require emergency interventions (3). In this report, we presented an extremely rare case of laryngocele which causes airway obstruction.

CASE REPORT

A 35-year-old male was admitted due to hoarseness, progressive respiratory distress and swelling at the right side of the neck for three years. The patient did not have a history of smoking, weight loss, trauma or significant family history. Physical examination revealed a 3 × 3 cm wide swelling at the 1/3 anterior part of the neck (Fig. 1A). Swelling was enlarged with Valsalva manoeuvre or coughing. The lesion was soft and fluctuating. The endoscopic examination revealed a swelling that started from the vallecula and extending to the right ventricle filling the right sinus pyriformis. A computerised tomography was performed that revealed an air-filled sac at the right part of the neck (Fig. 1B). The patient was operated under general anaesthesia. The mass was dissected from the surrounding tissue until the entrance of the laryngeal lumen and excised (Fig. 2). Direct laryngoscopy was performed, and disappearance of the mass was noticed. The patient did not have any complaint at the follow-up period.

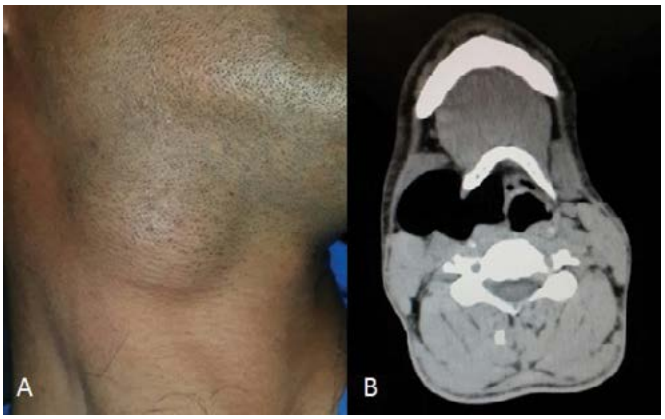

Fig. 1A: The appearance of the mass located at the right neck region. 1B: An axial CT showed air-filled sac at the right part of the neck.

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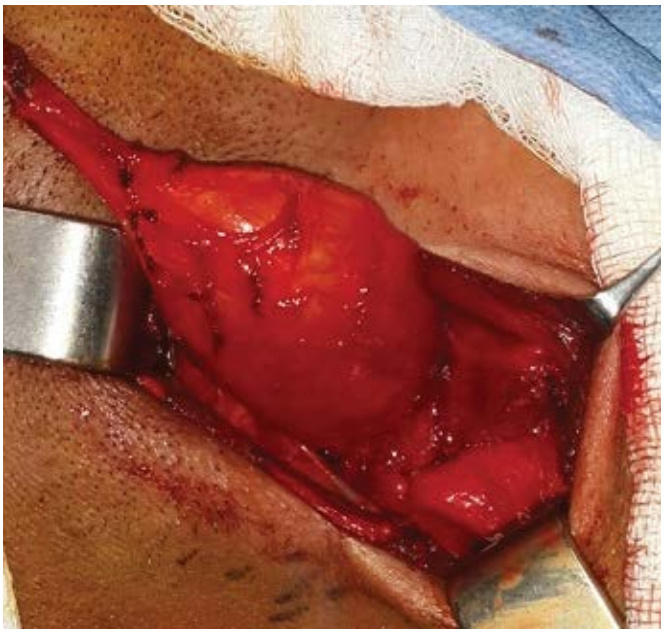

Fig. 2: Intraoperative appearance of the mass.

DISCUSSION

Laryngocele, which is in connection with laryngeal lumen, is an abnormal dilatation of the laryngeal saccule containing air (4). The incidence of laryngocele is estimated to be 1 per 2.5 million population per year. It is five-times more frequent in males than in females. Most cases occur in the 5th decade (5). Three types of laryngoceles are identified: internal, external and the mixed types. External laryngocele extends between the false vocal cord and the thyroid cartilage. Internal laryngocele is confined to the larynx and does not pierce the thyrohyoid membrane. A combined or mixed type includes both internal and external components of the laryngocele (3).

Symptoms depend on the type and size of the laryngocele. Most commonly laryngoceles are asymptomatic. Clinically patients may present with dyspnoea, hoarseness and cough. It is usually a lateral neck mass that enlarges with increased intralaryngeal pressure (6). There are three main aetiologic causes: congenital factors, mechanical obstruction and increased intralaryngeal pressure. Hobbies involving blowing or jobs such as trumpet playing or glass blowing can cause laryngoceles as blowing increases intralaryngeal pressures (7). Particularly, an external laryngocele presents as a lateral neck mass occurring during swallowing. Moreover an internal laryngocele presents with hoarseness due to interference with vocal cords. Also, an internal laryngocele rarely can cause acute airway obstruction due to sudden enlargement (8).

The most useful diagnostic tools are CT and magnetic resonance image (MRI) scans. Magnetic resonance image shows relations between laryngocele and laryngeal structures and extralaryngeal soft tissues (5).

When a laryngocele becomes symptomatic complete excision or marsupialisation can be performed by an endoscopic or an external approach. The choice of the approach is based on the type and size of the lesion. In cases of large or external laryngocele, an external approach is preferred, whereas an internal laryngocele can be operated with an endoscopic technique with the use of a CO_2 laser (9).

In conclusion, the laryngoceles are rarely symptomatic but because of their potential to cause sudden airway obstruction, they deserve serious attention.

REFERENCES

1. Marcotullio D, Paduano F, Magliulo G. Laryngopyocele: an atypical case. Am J Otolaryngol 1996; **17:** 345–8.
2. Cassano L, Lombardo P, Marchese-Ragona R, Pastore A. Laryngopyocele: three new clinical cases and review of the literature. Eur Arch Otorhinolaryngol 2000; **257:** 507–11.
3. Canalis RF, Maxwell DS, Hemenway WG. Laryngocele: an updated review. J Otolaryngol 1977; **6:** 191–9.
4. Dursun G, Ozgursoy OB, Beton S, Batikhan H. Current diagnosis and treatment of laryngocele in adults. Otolaryngol Head Neck Surg 2007; **136:** 211–15.
5. Balasubramanian T. Laryngocele: a review. Online J Otolaryngol 2015; **5:** 32–7.
6. Verret DJ, DeFatta RJ, Sinard R. Combined laryngocele. Ann Otol Rhinol Laryngol 2004; **113:** 594–6.
7. Amin M, Maran AG. The aetiology of laryngocele. Clinotolaryngol Allied Sci 1988; **13:** 267–72.
8. Bentyman W, Haidak GL, Taylor M. Laryngocele: a report of fatal case. New Engl J Med 1959; **260:** 1025–7.
9. Martinez Devesa P, Ghufoor K, Lloyd S, Howard D. Endoscopic CO2 laser management of laryngocele. Laryngoscope 2002; **112:** 1426–30.

CASE REPORT

Use of a Removable Silicone Bung for Increased Seal and Retention of an Obturator in the Prosthetic Rehabilitation of a Unilateral Maxillary Defect: A Clinical Case Report

A Paryag, T Peters, P Seeratan, J Lowe, R Rafeek

ABSTRACT

Patients with acquired maxillary defects differ from those with congenital defects due to the abrupt alteration in physiologic processes associated with surgical resection of the maxillae. The post-surgical effects affect the form and function of the normal stomatognathic system. The quality of life of the patient is therefore reduced as the end state can be particularly severe. An obturator is a prosthesis that closes a palatal defect in both dentate and edentulous patient. Early management with an option such as this is therefore important in retaining function and enhancing aesthetics and thus possibly safeguarding the patient's self-esteem. The report outlines the fabrication of a maxillary obturation with a removable silicone bung that enables eventual replacement of the bung limiting the exposure to potentially virulent fungal and bacterial growths that can be harmful to immune compromised patients. While not without its disadvantages, this simple addition may make it possible for a better fit of intermediate acrylic obturators in a cost-effective manner. It could be especially useful where the injection moulded polymer obturator is not available or is difficult to obtain due to the prohibitive cost.

Keywords: Maxillary defect; maxillary obturator; maxillofacial prosthodontics; maxillary surgery defect rehabilitation

INTRODUCTION

Maxillary defects are created by surgical treatment of benign or malignant neoplasms, as well as congenital malformation and trauma, and their occurrence is also associated with the enucleation of maxillary cysts (1). Squamous cell carcinomas account for two-thirds of the malignant neoplasms of the upper gingiva and hard palate. Lesions in these areas account for 1%–5% of total occurrence in the oral cavity (1). Adjacent structures are vulnerable to metastasis during the confirmation of the diagnosis. With this eventuality, the recommended treatment for these types of lesions is alveolectomy, palatectomy, and partial or total maxillectomy. These treatment outcomes depend on the location and aggressiveness of the actual lesion, its histiotype, patient's age and general health status (1). Patients with acquired maxillary defects differ from those with congenital defects due to the abrupt alteration in physiologic processes associated with surgical resection of the maxillae (1). The post-surgical effects affect the form and function of the normal stomatognathic system. The quality of life of the patient is therefore reduced as the end state can be particularly severe (2). Patients can experience hypernasal speech, regurgitation of food or fluid into the nasal cavity, and impairment of mastication and deglutition. The facial contour of the patient can also be affected, particularly when it involves one or both sides of maxilla with or without associated paranasal sinuses (2). 'Rehabilitation of these acquired maxillary defects can be accomplished by using various types of microvascularised flaps for smaller defects or by prosthetic means for larger defects' (2).

An obturator is a prosthesis that closes a palatal defect in both dentate and edentulous patient (3). Early

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management with an option such as this is therefore important in retaining function and enhancing aesthetics and thus possibly safeguarding the patient's self-esteem (4).

The choice between options is influenced by the site, size, aetiology, severity, age and the patient's wishes. However, the success of the prosthodontic option depends upon treatment planning and collaboration between the surgeons and prosthodontist, with consideration of patient expectations. 'Prosthodontic rehabilitation of total or partial maxillectomy in patients includes the separation of oral and nasal cavities to allow adequate deglutition and articulation, support for the orbital contents to prevent enophthalmos and diplopia, support of the soft-tissue to restore the midfacial contour and an acceptable aesthetic result' (5). 'It is positioned within the closure of a congenital or acquired tissue opening, primarily located in the hard palate and/or contiguous alveolar or soft-tissue structures' (6).

The time period that has passed from surgery decides the classification of the obturator. A patient can therefore receive an immediate surgical obturator (feeding plate); temporary or interim obturator and definitive obturator (7).

After surgical treatment, an immediate surgical obturator is placed right away. This is used to reduce any of the complications to be had (8). Soft tissue is supported by this obturator, and the contracture of the resulting scar minimised. There is also a reduction of disfigurement (maintains some measure of palatal contour) while protecting the surgical packing from contamination (9). A regular diet can then be resumed, and the wound remains protected from trauma. Pressure is maintained either directly or indirectly on any split thickness skin graft (10). Furthermore, it restores speech to a reasonable level and obviates the use of naso-gastric tubes. 'They can also be used to correct lip and cheek contour and to reduce the flow of exudates into the mouth' (11).

According to Keyf (3) 'the temporary or interim obturator is constructed from a cast poured from a post-surgical impression. A closed but hollow bulb may extend into the defect replicated on the palatal region and soft tissue areas'.

'The definitive obturator is fabricated approximately six months after surgery from a post-surgical maxillary cast, at a time when the surgical site has completely healed and minimal dimensional changes are unlikely'. As such this type of obturator has a metal framework, which acts as the palate and supports the teeth and a closed hollow bulb (12).

The obturator design hinges upon the specific surgical defect, which would range from the very undemanding to an extensive defect. Thus, a universal classification of maxillectomy defects results in the adjustment in objective in the design of the prosthesis. According to one systematic review of literature describing a search for such classifications, there is no universally accepted classification system that serves both the surgeon and the prosthodontist simultaneously (13). Individual criteria necessary for such a classification were described and it was found that those from the prosthodontist perspective considered treatment planning for the defect long after surgery. The criteria included dental status, oroantral or nasal communication, contiguous structures involvement, superior–inferior extension, anterior–posterior extension and medial–lateral extension.

Knowing the dental status allows for treatment planning, tooth facilitated or implant retention of the prosthesis, while the absence of an air space (nasal or antral) communication removes the difficulty of the design in providing physical separation between the oral cavity and other spaces. The involvement of contiguous structures presents a difficulty in planning the extent and retention of the intra-oral prosthesis over a wider area of skeletal and soft tissue. The superior extension allows for determining the ideal height necessary for peripheral seal, while anterior–posterior extension attracts issues of re-establishing esthetics, namely, lip support, midfacial support, lip competency and also pharyngeal and soft palate functioning. Finally, medial–lateral extension dictates the amount of surface area available for support and retention, in cases where the dental status is poor and implants are precluded. It also indicates the amount of tissue that the prosthesis is to cover.

Various materials have been used for the fabrication of obturators including hard acrylic resins such as PMMA, flexible or thermoplastic acrylic resins. In addition acrylic soft liners and silicone soft liners are used (14, 15). As early as the 1930s, a compression moulding technique has been used widely throughout the world for making intraoral removable prosthesis. There is no doubt that this technique has served patients for many years, but it is disadvantageous with regard to some aspects such as polymerisation shrinkage, residual monomer, discomfort due to the hardness of the material and the inability to atraumatically engage undercuts. Patients have already undergone surgery, and many experience mental and physical agony. In addition, the residual tissue is also highly compromised. The challenge for the prosthodontist is to utilise

the remaining tissues in the best way possible by using a material that can easily engage the retentive undercuts, avert the disadvantages of acrylic material and be easily acceptable to the patient (15).

Rehabilitation of course is not without possible negative outcomes. However, these can be outweighed by the positive aspects of providing an obturator prosthesis to the patient. One such disadvantage is the colonisation of the obturator prosthesis by microorganisms with the possibility of prosthesis-related infection. At the delivery of any prosthesis management of this possibility is of paramount importance. It becomes ever more critical with the rehabilitation of compromised tissues. As mentioned previously, various materials are used to fabricate obturator prostheses. *Candida* species colonisation presents a problem for soft liners and silicone (16) while in addition to the presence of the common Streptococci, microflora of nose and sinus such as *Staphylococcus* spp, corynebacteria, *Haemophilus* spp. and neisseriae (17, 18) are also present in the biofilm. These can be potentially virulent in the immunocompromised (16, 19).

CASE REPORT

Patient history

A 24-year-old female student of Indo-Trinidadian descent with an unremarkable medical history presented to the UWI School of Dentistry for prosthetic rehabilitation following a low left hemimaxillectomy and level one left neck dissection in the removal of a high-grade mucoepidermoid carcinoma (MEC) with nodal involvement (2012). She is a non-drinker and non-smoker. Prior to this, the patient underwent left maxillary resection and cryosurgery for an 'Ameloblastoma' (2007) (report was unavailable). There was suspicion of recurrence; however, the diagnosis of MEC was confirmed upon histopathological review of the surgical specimen removed at second admission. The specimen included left maxillary tuberosity with upper third molar, pterygoid plates and left sinus with polyps. The submental and left submandibular salivary glands were also removed. An immediate surgical obturator with soft reline was fitted after maxillectomy. Subsequently, several interim obturators were fabricated (Figs 1–3 show one such obturator that was fabricated 2 months after the second surgery), the most recent obturator was fabricated in 2013; however, the patient underwent radiotherapy in that same year followed by removal of a recurrent/secondary mass in 2014 and thus required yet another replacement.

Clinical findings

At the time of referral to the prosthodontic department, the patient was not actively wearing an obturator. The patient commented that intermediate obturators provided for her never completely prevented the inflow of from the oral cavity to nasal air space and as such 'practiced to keep liquids down'. The past obturators were generally stable and comfortable to the patient. However, post-radiotherapy and a third surgery, the obturator in use became ill fitting and irritating to the inflamed and then healing soft tissues.

The patient required only prophylaxis and minor restorative work prior to the commencement of fabricating another obturator. Figures 4 and 5 show views of the defect at presentation.

Fig. 1: Interim obturator fabricated 2 months after surgery—outer surface view.

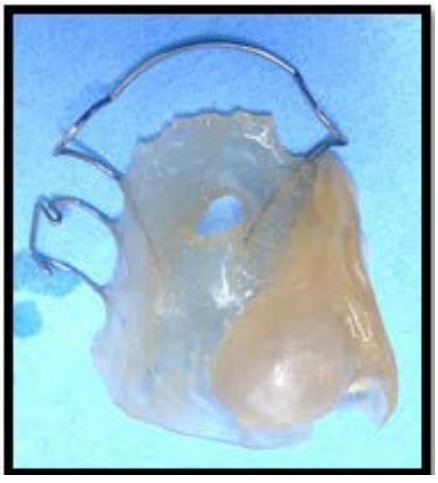


Fig. 2: Interim obturator fabricated 2 months after surgery—inner fit surface view.

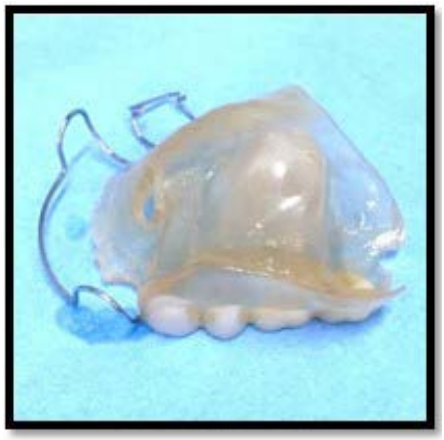


Fig. 3: Interim obturator fabricated 2 months after surgery—side view.

Fig. 4: Lateral view of defect.

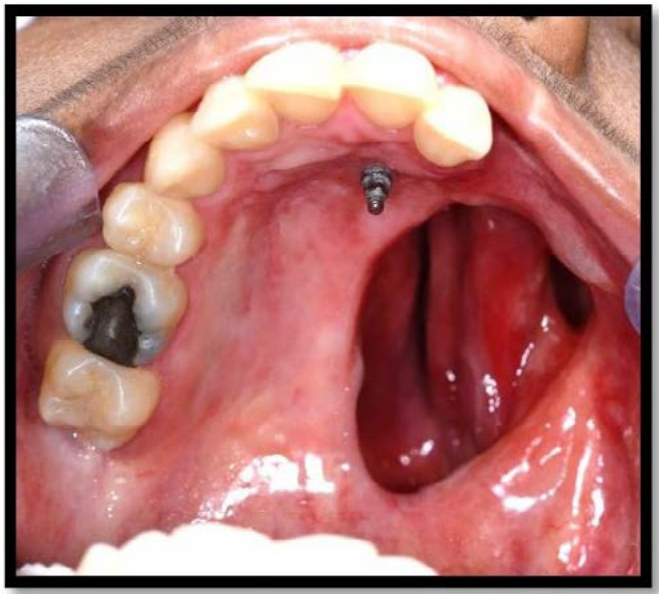


Fig. 5 : Maxillary occlusal view of defect.

DISCUSSION

Post-surgically, the patient is seen to have a resultant Type II defect according to the classification by Okay *et al.* (20) or a Vertical Type II and Horizontal Type II according to Brown *et al*. (21). The intraoral view shows that the defect does not involve the premaxilla. However, the left canine was involved. It is seen not to cross the midline and the vertical extent does not include the orbit. Of the two classifications, the latter seems to more precisely define this defect (Table).

An impression of the defect was made using polyvinyl siloxane putty in a stock tray and a light body wash placed into the defect and over the occlusal surfaces of all teeth present, secondary to placing ribbon gauze into the defect. The resultant impression accurately captured the vertical and horizontal extensions of the defect, and all excess materials were removed on withdrawal of the ribbon gauze.

A study cast was poured, upon which an extension of the proposed obturator into the defect was delineated (Fig. 6). Undercut areas were blocked out and the cast duplicated. The initial temporary acrylic base was fabricated and occlusal rims added to this.

A traditional record of the occlusion was obtained. Initially, there was difficulty in retaining the baseplate against the defect to complete this step. As such an

Table: A pair of classifications for maxillectomy defects that may be found to be the most mindful of prosthodontic considerations (20, 21)

Contributor	Classification
Okay *et al.* (20)	Ia—Defects of any portion of hard palate excluding tooth bearing maxillary alveolus Ib—Defects of premaxilla or any portion of alveolus or dentition posterior to canines II—Defects include any portion of hard palate, alveolus and only one canine tooth. They also include transverse palatectomy involving less than 50% of hard palate III—Defects include resection of any portion of hard palate, alveolus and both canine teeth. They also include transverse palatectomy involving greater than 50% of the hard palate IV—Subclasses f and z denote involvement of orbital floor and any portion of zygoma, respectively
Brown *et al.* (21)	Vertical I—Maxillectomy not causing an oronasal fistula II—Maxillectomy not involving orbit III—Maxillectomy involving orbital adnexae with orbital retention IV—Maxillectomy with orbital enucleation or exenteration V—Orbitomaxillary defect VI—Nasomaxillary defect Horizontal I—Palatal defect only, not involving dental alveolus II—Less than or equal to 1/2 unilateral III—Less than or equal to 1/2 bilateral or transverse anterior IV—Greater than 1/2 maxillectomy

Adams clasp was inserted in order to increase retention while adjusting the occlusal rim.

During final processing, the undercuts within the defect were again blocked out on the working cast and the acrylic extended only partially into the defect.

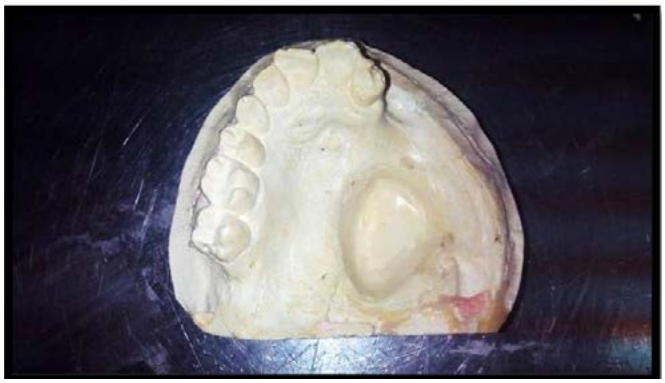


Fig. 6: Study cast showing full extent of palatal defect.

Post-processing, the first duplicate cast was scored within 3 mm of the borders of the defect to produce a minimal undercut (Fig. 7). Silicone was injected into this defect, and the processed obturator seated carefully with pressure until the silicone was completely set.

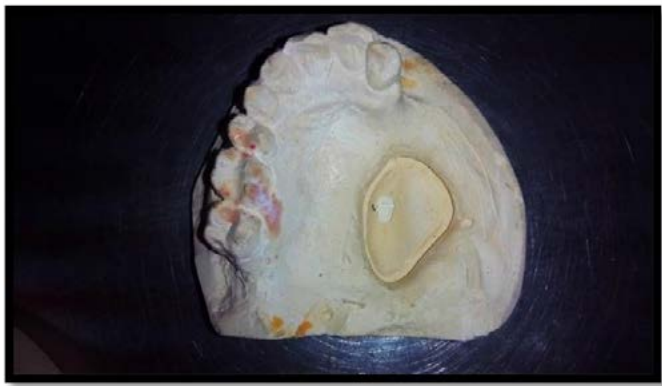


Fig. 7: Study cast showing defect after partially blocking out undercut in order to fabricate the obturator.

The resultant final prosthesis therefore consisted of a hard acrylic base that adapted closely to the dentition and palatal tissues (Fig. 8).

The method of fabrication allows the bung to be removed for cleaning such as with chlorhexidine gluconate (short period of soaking). This should limit the growth of any potentially infectious microorganisms.

Upon writing this report, the patient had been wearing the new obturator for a period of six (6) weeks. There was a complaint of muscle stiffness while wearing the prosthesis. However, she has commented on the increased comfort of fit and adequacy of the seal produced as compared to her previous obturator, which did not have the silicone bung (Figs 9–14 show the obturator in situ in patient's oral cavity with replacement of teeth and improvement of esthetics). Minor adjustments to the acrylic were made to remove excessive width of the flange area as well as sharp areas on the tissue bearing side. Additions to the tooth bearing acrylic in the region of the canine as well as removal of the anterior (south end) clasps were done to improve aesthetics (patient's wish), although at the expense of retention.

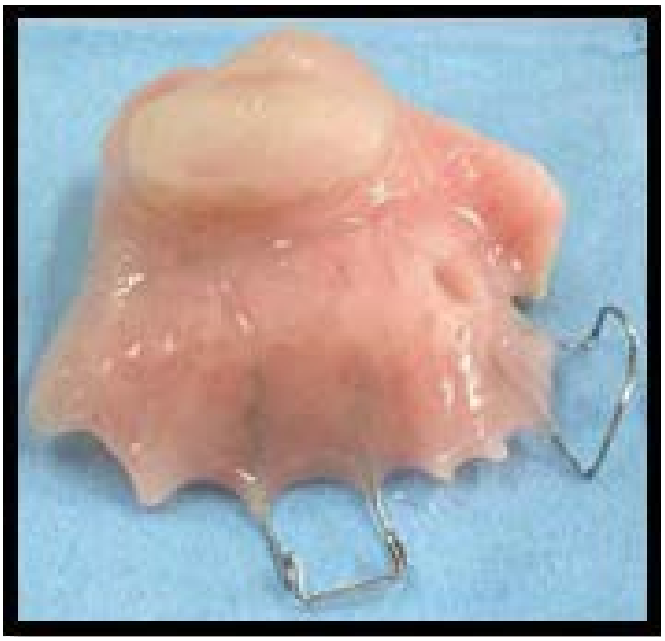
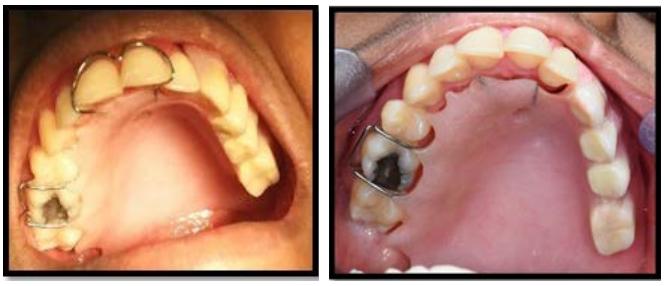


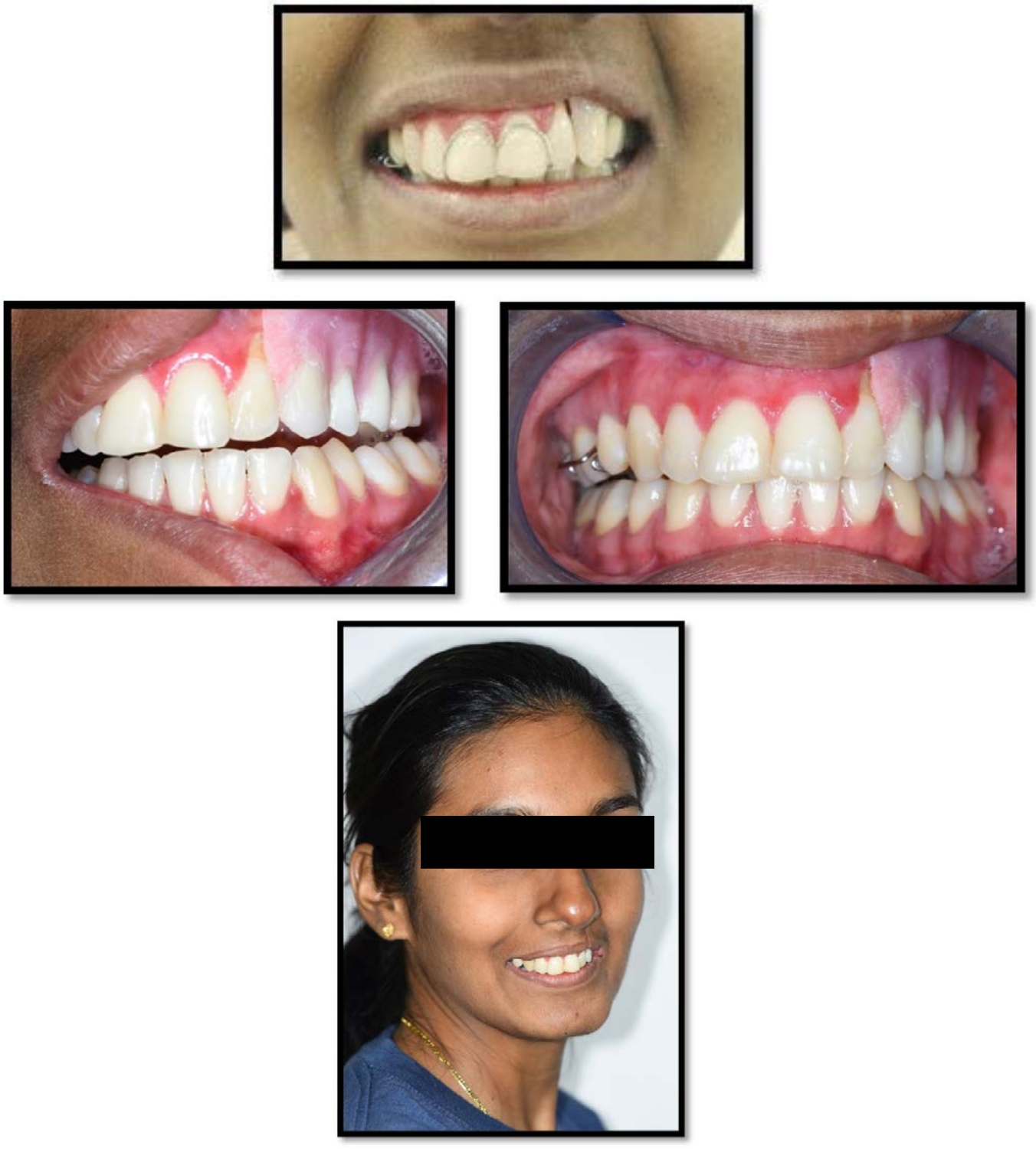
Fig. 8: Obturator showing silicone bung.



Figs. 9 and 10: Obturator in situ—palatal view.

CONCLUSION

The use of the silicone bung served to increase retention and provide an adequate seal while maintaining comfort for the patient. While not without its disadvantages, this simple addition may make it possible for better fit of intermediate acrylic obturators in a cost-effective manner. It could be especially useful where the injection moulded polymer obturator is not available or is difficult to obtain due to prohibitive cost.



Figs. 11–14: Obturator in situ—anterior views.

REFERENCES

1. Tirelli G, Rizzo R, Biasotto M, Di Lenarda R, Argenti B, Gatto A, et al. Obturator prostheses following palatal resection: clinical cases. Acta Otorhinolaryngol Ital. 2010; **30:** 33–9
2. Garhnayak M, Garhnayak L, Kar AK, Chittaranjan B. Prosthetic rehabilitation of a unilateral maxillary defect with an intermediate obturator. Indian J Dent Res. 2010; **2:** 378–82.
3. Keyf F. Obturator prostheses for hemimaxillectomy patients. J Oral Rehabil. 2001; **28:** 821–9.
4. Popli S, Parkash H, Bhargava A, Gupta S, Bablani D, Kar AK. A two-piece sectional interim obturator. A clinical report. J Prosthodont. 2012; **21:** 487–90.
5. Singh M, Bhushan A, Kumar N, Chand S. Obturator prosthesis for hemimaxillectomy patients. Natl J Maxillofac Surg. 2013; **4:** 117–120.
6. The glossary of prosthodontic terms. J Prosthet Dent. 2005; **94:** 10–92
7. Wiens JP. Acquired maxillofacial defects from motor vehicle accidents: statistics and prosthodontic considerations. J Prosthet Dent. 1990; **63:** 172–81.
8. Türkaslan S, Baykul T, Aydýn MA, Ozarslan MM. Influence of immediate and permanent obturators on facial contours: a case series. Cases J. 2009; **2:** 6.
9. Shambharkar VI, Puri SB, Patil PG. A simple technique to fabricate a surgical obturator restoring the defect in original anatomical form. J Adv Prosthodont. 2011; **3:** 106–9.
10. Juntavee N. Prosthetic rehabilitation of maxillectomy patient. Kaen Dent J. 1998; **1:** 93–9.
11. Meenakshi A, Shah D. The obturator prostheses for maxillectomy. SRM J Res Dent Sci. 2012; **3:** 193–7.
12. Singh M, Bhushan A, Kumar N, Chand S. Obturator prosthesis for hemi maxillectomy patients. Natl J Maxfac Surg. 2013; **4:** 117–20.
13. Bidra AS, Jacob RF, Taylor TD. Classification of maxillectomy defects: a systematic review and criteria necessary for a universal description. J Prosthet Dent. 2012; **107:** 261–70.
14. Tannamala PK, Pulagam M, Pottem SR, Karnam S. Flexible resins in the rehabilitation of maxillectomy patient. Indian J Dent Res 2012; **23:** 97–100.
15. Batra P. An obturator with a soft touch: a case report. Int J Sci Technol Res 2001; **2:** 13–18.
16. Pereira-Cenci T, Del Bel Cury AA, Crielaard W, Ten Cate JM. Development of Candida-associated denture stomatitis: new insights. J Appl Oral Sci. 2008; **16:** 86–94.
17. Mc Cracken AW, Land GA. Basic sciences. In: M Gleeson ed. Scott-Brown's Otolaryngology. Oxford: Butterworth-Heinemann; 1997.
18. Weston V. Microbiology. In: NS Jones ed. Scott-Brown's Otolaryngology. Oxford: Oxford University Press. 2008.
19. Wargo MJ, Hogan DA. Fungal-bacterial interactions: a mixed bag of mingling microbes. Curr Opin Microbiol 2006; **9:** 359–64.
20. Okay DJ, Genden E, Buchbinder D, Urken M. Prosthodontic guidelines for surgical reconstruction of the maxilla: a classification system of defects. J Prosthet Dent 2001; **86:** 352–63
21. Brown JS, Rogers SN, McNally DN, Boyle M. A modified classification for the maxillectomy defect. Head Neck 2000; **22:** 17–26.

CASE REPORT

Munchausen Syndrome by Proxy Presented with Multiple Bone Fractures

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ABSTRACT

Munchausen Syndrome by Proxy (MSBP) is about the caregiver (perpetrator) who abuses and reveals signs of a disease of the victim because of her/his own psychological needs such as attracting and getting attention. Munchausen Syndrome by Proxy is a complicated form of. child abuse form, which may cause important physiological and psychological effects and has mortality risk in the long-term period. In a study, 93% of the cases found the perpetrator to be the mother, followed by the female babysitter and the father. Usually, the symptoms disappear during the mother's or the caregiver's absence. The more frequently reported findings are bleeding, apnoea, nutritional problems, vomiting, diarrhoea, convulsion, cyanosis, asthma, allergies, fever and pain. In studies conducted, the death rate is reported to be between 6% and 10% in the victims. Common opinion is that if no response is received from the practitioners in the treatment or if insufficient progress is shown (e.g., in a six months period), victims should be taken from their environment and should not be given back to their families. Here, we are representing a female perpetrator of MSBP who gives damage to her children by traumas like recurrent bone fractures and tissue damage, and as a result of a forensic notification, whose children are taken away and given to foster care.

Keywords: Bone fracture, child abuse, Munchausen syndrome by proxy

INTRODUCTION

Munchausen Syndrome by Proxy (MSBP) is about the caregiver (perpetrator) who abuses and reveals signs of a disease of the victim because of her/his own psychological needs like attracting and getting attention. Munchausen Syndrome by Proxy may cause physiological and psychological effects and has mortality risk (1). Here, we present a case of MSBP by a mother who injures her children by producing traumas such as recurrent bone fractures, and result of a forensic notification, whose children were taken away and placed in foster care.

CASE REPORT

A 34-year-old woman with six children brought her 5-year-old son to the emergency room with a complaint of right leg pain. She mentioned that her son fell off a broken ladder in their house. In his physical examination, there was sensitivity in the right leg and the complaint of pain that occurred with moving. In conventional radiography of bilateral lower extremity, a fracture in the right tibia was detected; the right leg was encased in plaster and the boy was discharged with follow-up recommendation. About two weeks later, she brought her 7-year-old son to the emergency room with difficulty in walking, bruises and scars on his legs his legs. She stated that her son fell down the stairs. As a result of the conducted analyses, no fractures were detected, though new bruises made the orthopaedist suspicious. He was admitted to the orthopaedic clinic and was taken under treatment. Three months later, the same woman brought her 12-year-old child to the emergency room with similar complaints. The orthopaedist, suspecting child abuse, requested psychiatric consultation and called the hospital police. In her psychiatric examination, she was conscious, fully cooperated to time-place-person. Her affect was blunted, and speech was in the form of question and answer. No perception and/or thought content

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abnormality was seen. Her intelligence quotient score was 83, implementing as dull intelligence level. The impression from the clinical observation was borderline mental capacity. In her anamnesis, she reported that her husband was a long-distance driver, and because of that she has been spending most of the days of the year alone with her children. When the past hospital records about the family were investigated, it was found that there were many emergency appeals about her children. On physical examination, the children had scars, and X-rays of the legs revealed older fractures from different time intervals. Further anamnesis revealed that she had been causing fractures and bruises to her sons using her husband's truck wedge. It was also learned that she had been informing her husband about the illnesses and hospitalisation of the children prompting him to return home earlier than usual and spend more time together. This situation was reported to the forensic units; and the children were taken away from their parents and placed in a state institution where they will be taken care of.

DISCUSSION

Munchausen Syndrome by Proxy, according to the types of other child abuse, is quite common and incidence studies in children under the age of 16 years reported a rate of 0.4/100 000, whereas under the age of one year, the rate was 2–2.8/100 000 found. However, the actual incidence is higher than this estimated amount (2–5). One study showed that in 93% of cases the perpetrator was found to be the mother, similar with our case, and according to the order of frequency, the other implementers were babysitter and the father (5). In the MSBP, the symptoms, physical findings and laboratory results can be extraordinary, and usually inconsistent with the patient's situation and history. The relationship between the disease's clinical findings and prognosis cannot be established.

Patients usually do not respond to the treatment, or even if they do at the beginning, symptoms recrudescence afterwards (6). Usually, the symptoms disappear during the caregiver's absence (7). Due to the continuous applications to emergency service, diagnosis may go unnoticed. Most of the time, the doctors involuntarily get involved to this period with performing unnecessary diagnostic and interventional procedures or prescribing drugs that have a high potential for side effects. Victims' 75% of morbidity was found to be as a result of initiatives that are performed by the doctors in the hospital (3). MSPB is a form of child abuse, which has quite a high risk of morbidity and mortality. In studies conducted, the death rate is reported to be between 6% and 10% in the victims. In addition to this, if the victim is poisoned or suffocated, the death rate increases up to 33% (3, 5). In this syndrome, it is hard to understand the underlying psychopathology of the practitioner. It should not be forgotten that there is no diagnostic test or psychological profile that excludes or supports the diagnosis of the MSBP. In the MSBP, narcissistic fragility and borderline personality traits are frequent; also passive-dependent, hysterical personality traits, different sadomasochistic behaviours, mild or borderline mental retardation, compliance issues and depression have been found (8). In our case, the patient was thought to have a borderline mental capacity. This situation may have influenced the mother's executive functions negatively, so that she could not find an alternative solution to absence of her husband in order to spend more time with him.

It is known that MSBP has a high relapse risk (9). In our case, a rare type of MSBP is studied that occurred with repetitive bone fractures in multiple family members. It has been noticed that the mother was trying to draw attention by creating these bone fractures and intended to spend more time with her husband. Another MSBP case diagnosed on suspicion after 11 months of having repetitive humerus, femur and tibia fractures and skin lesions in a 3-year-old victim. The mother created disease symptoms on the child in order to maintain her own psychological needs and tried to organise her husband and spend more time with him (10). There are limited number of studies about long-term follow-ups of the victims who were exposed to this childhood abuse. In these studies, it is reported that starting from childhood and continuing in adulthood, the victims may develop problems like insecurity, being opposed and oppositional defiant disorder, post-traumatic disorder, attention disorders, attachment and social relationship problems, avoiding medical treatment and low self-esteem. What is more, it is found that some of the victims developed 'Munchausen Syndrome' in their adolescence and young adulthood (1, 11). In our case, information on the progress of the victims could not be obtained because they were placed in foster care. Making a diagnosis is usually hard; if any case is considered to be MSBP, it should be approached in a suspicious manner to each case. It is important to remember that the first condition of the diagnosis is to be suspicious; if there are any diagnosis of suspected MSBP cases, a retrospective review of them and their siblings should be performed.

As we did in our case, investigating the hospital records of the patient's siblings is important for the

diagnosis. A collaborative work of a multidisciplinary team provides guidance for the diagnosis of MSBP. Priority should be given to the victim's safety while collecting the necessary evidence for the diagnosis, and child's life should not be at more risk. Common opinion is that if no response is received from the practitioners in the treatment or if insufficient progress is shown (*eg*, in 6 months period), the victims should be taken from their environment and should not be given back to their families. Due to the high risk of recurrence in the children who are given back to their families, close monitoring and follow-ups are recommended (3, 9). The children in our case were taken from their families and have been taken under the state protection. We cannot give further information about the current status because we could not follow the family further.

REFERENCES

1. Sharif I. Munchausen syndrome by proxy. Pediatr Rev 2004; **25:** 215−16.
2. McGovern M, Smith M. Causes of apparent life threatening events in infants: a systematic review. Arch Dis Child 2004; **89:** 1043−8.
3. Galvin HK, Newton AW, Vandeven AM. Update on Munchausen syndrome by proxy. Curr Opin Pediatr 2005; **17:** 252−7.
4. Hymel KP, the Committee on Child Abuse and Neglect. Distinguishing sudden infant death syndrome from child abuse fatalities. Pediatrics 2001; **107:** 437−41.
5. Terry L. Fabricated or induced illness in children: nurses focus on protecting children but they also need support and protection from harm during and after an investigation, as Louise Terry explains. Paediatric Care 2004; **16:** 14−18.
6. Foto-Özdemir D, Yalçın SS, Zeki A, Yurdakök K, Özusta Ş, Köse A et al. Munchausen syndrome by proxy presented as recurrent respiratory arrest and thigh abscess: a case study and overview. Turk J Pediatr 2013; **55:** 437−43.
7. Squires JE, Squires Jr RH. Munchausen syndrome by proxy: ongoing clinical challenges. J Pediatr Gastr Nutr 2010; **51:** 248−53.
8. Bouden A, Krebs M, Loo H, Olie J. Munchausen syndrome by proxy: a challenge for medicine. Presse medicale (Paris, France: 1983) 1996; **25:** 567−9.
9. Rosenberg DA. Web of deceit: a literature review of Munchausen syndrome by proxy. Child Abuse Neglect 1987; **11:** 547−63.
10. Sugandhan S, Gupta S, Khandpur S, Khnna N, Mehta M, Inna P. Munchausen syndrome by proxy' presenting as battered child syndrome: a report of two cases. Int J Dermatol 2010; **49:** 679−83.
11. Bools C, Neale B, Meadow S. Follow up of victims of fabricated illness (Munchausen syndrome by proxy). Arch Dis Child 1993; **69:** 625−30.

CASE REPORT

Encrusted Suprapubic Catheter in Urethral Injury with Pulmonary Embolism: What To Do?

AH Hamzah[1], AS Adnan[2,3], TH Mallhi[4], M Nor Gohar Rahman[5], YH Khan[6,7], AH Khan[8]

ABSTRACT

Suprapubic catheters (SPCs) have advantages over urethral catheters with respect to decreased bacteriuria and greater patient acceptability. Catheter encrustation is one of the most common intricacies of SPC and is treated with extracorporeal shock wave lithotripsy and endoscopy lithotripsy. We reported a case of blocked, encrusted SPC, along with urethral injury, that was further complicated by pulmonary embolism. All conventional techniques to treat encrustation were not suitable due to worsening of urethral injury and risk of bleeding as the patient was taking anti-coagulants. Therefore, we adopted a safe and quick procedure by which a 5 mm 0° cystoscope was inserted and the encrusted part of the catheter was fragmented, using artery forceps, leading to removal of the catheter without jeopardy to the catheter track and patient's safety.

Keywords: Catheter, pulmonary embolism, suprapubic catheter, urethral injury, urology.

INTRODUCTION

Urinary bladder catheters are used for urinary drainage or as a means to collect urine for measurement. Suprapubic catheters (SPC) are the most invasive catheter and require a surgical procedure for placement and insertion through the abdominal wall into the bladder, either intraoperatively in association with another surgical procedure or percutaneously (1). Suprapubic catheterisation has advantages over urethral catheterisation with respect to decreased bacteriuria and greater patient acceptability (2). Avoidance of urethral strictures, decreased level of nursing care, preserved sexual function, prevention of penile pressure necrosis, early post-surgical ambulation, lower infection rates, ease of bladder training, shorter hospitalisation stays and decreased postoperative infections are some other additional benefits of SPC (3). However, SPC is also accompanied by several minor and rare, major complications like bladder perforation, catheter migration, bleeding and blockage. Encrustation is one of the most common causes of catheter blockage that is usually treated with extracorporeal shock wave lithotripsy (ESWL) and endoscopy lithotripsy (EL). Here, we reported a case of SPC encrustation with urethral injury, complicated with pulmonary embolism, for which the patient was taking anticoagulants. We described a new technique for the removal of SPC as the patient was not suitable for conventional methods of SPC removal due to anticoagulant therapy and underlying urethral injury.

CASE REPORT

A 45-year-old Malay male met with a motor vehicle accident and had sustained open book pelvic fracture. Micturating cystouretrogram showed bulbous urethral injury (Fig. 1) for which SPC was placed (Fig. 2).

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The patient also had T10-L1 fracture with neurological deficit up to L1 level. The patient underwent open reduction and internal plate fixation for pelvic fracture, and delayed repair was planned for urethral injury. Unfortunately, the patient developed deep vein thrombosis (DVT) after surgery, due to immobility, for which low-molecular-weight heparin was initiated. Later, the patient developed massive pulmonary embolism as a result of DVT, and warfarin was started to maintain international normalised ratio values within the range of 2–3. The patient was then discharged from hospital with SPC and was directed to change the catheter after every 2 weeks.

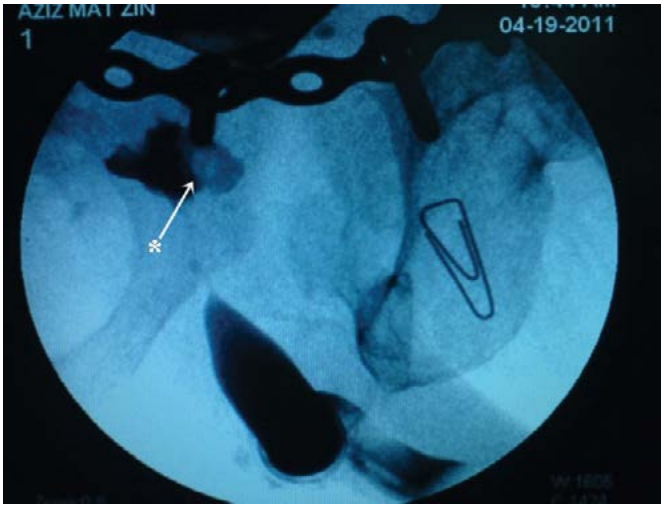

Fig. 1: Micturating cystouretrogram (MCUG) indicating bulbous urethral injury (*).

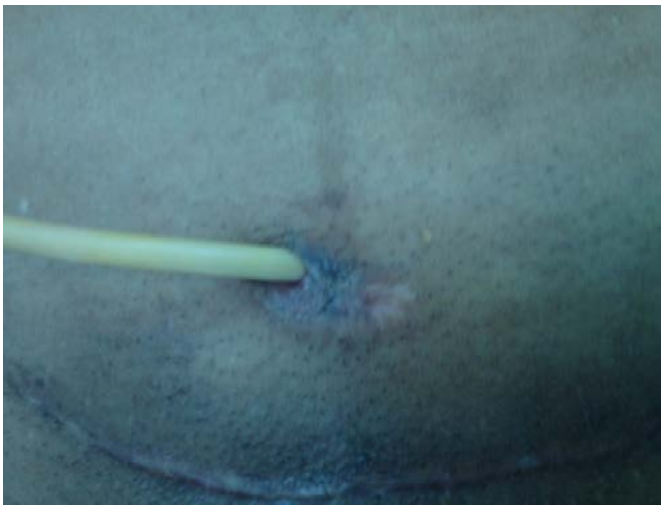

Fig. 2: Placement of a suprapubic catheter due to delay repair for ureteral injury.

One month later, the patient was referred to urology outpatient clinic due to failure of catheter removal. An X-ray demonstrated catheter encrustation (Fig. 3), while computed tomography scan of the pelvic region showed the presence of calcification ring on the catheter, suggesting encrustation.

All the conventional methods to treat encrustation were associated with high morbidity in this case, due to the use of anticoagulant therapy for pulmonary embolism. Repeated insertion of a lithotripter through the urethra was not suitable in our patient because of underlying urethral injury. So, we adopted a quick and safe technique of removing encrusted SPC in this high-risk patient. We admitted the patient and planned the procedure on the fifth day of admission. Warfarin was stopped and heparin was infused 3 days prior to the procedure with monitoring of activated partial thromboplastin time until its values were two times higher than normal. On procedure day, suprapubic tract was dilated by using artery forceps under spinal anaesthesia. Under the direct vision, a 5 mm 0° cystoscope was inserted and encrusted part of the catheter was fragmented using artery forceps, leading to removal of the catheter (Fig. 4). The catheter track was thoroughly washed and a new SPC was placed. Oral warfarin was started immediately, and the patient was discharged on the second day of procedure without any complication or bleeding episode.

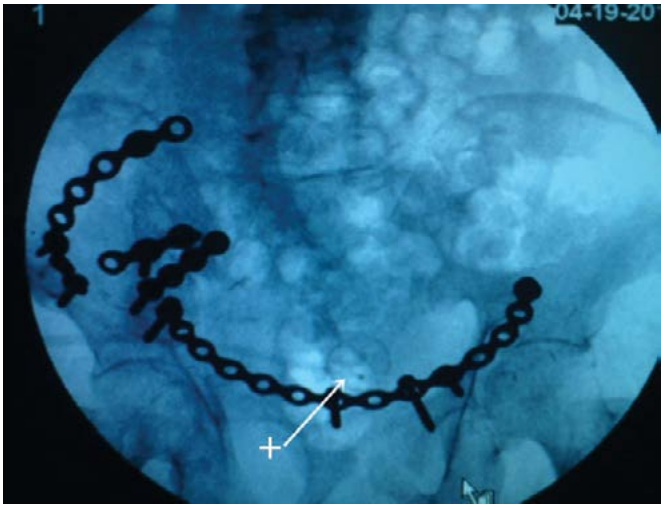

Fig. 3: MX-ray showing calcified ring suggesting encrusted catheter (+).

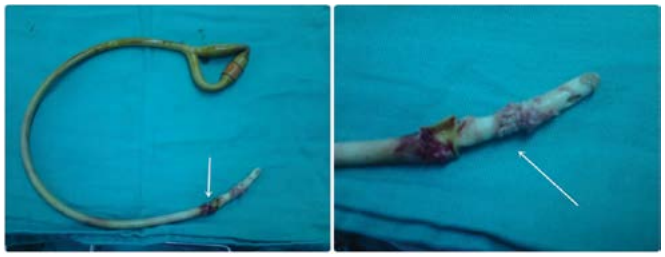

Fig. 4: Removed suprapubic catheter with calcified tip.

DISCUSSION

Urethral injury can be realigned by rigid cystoscopy in the early phase of injury or by delayed urethral repair after 6 weeks of injury, but in later case, placement of SPC is needed to bypass the urethral (4). In our case, delayed repair was planned because the patient had open book pelvic fracture with neurological deficit where prognosis is guarded. A long-term catheterisation was also planned, due to high risk of neurogenic bladder. Catheter entrapment is a potential complication and occurs due to balloon malfunction, faulty valve mechanism, malfunction of the inflation channel or crystallisation of fluid within the balloon (5).

Besides the advantages of SPC over urethral catheterisation, it also carries several complications like infection, bladder spasm, balloon malfunction and intraluminal or extraluminal catheter encrustation (2). The encrustation mechanism can be explained by the reason that the insertion of a catheter in body causes immune response, leading to formation of bacterial biofilm on the catheter. This biofilm secretes an extracellular matrix of bacterial glycocalyces and host proteins. Urinary crystals, such as struvite and calcium phosphate, are also incorporated into biofilm (6). Alkalisation of urine by urease enzyme, released by *Proteus mirabilis*, promotes crystallisation of struvite and calcium phosphate (7).

Under normal circumstances, encrustation of a catheter can be treated either with ESWL or EL. Canby-Hagino *et al*. described the use of intraluminal pneumatic lithotripsy for removal of intraluminal encrustation of the catheter; a pneumatic lithotripsy probe is inserted into the lumen of the catheter and advanced in a jackhammer-like fashion. This technique results in disruption of intraluminal encrustations and straightening of the tubes so that they can be removed in an atraumatic manner (8). Kunzman *et al*. reported that use of ESWL delivered at 8 kV level causes fragmentation of encrusted balloon (9). Christopher Ho reported a case in which an encrusted urethral catheter was fragmented by using a lithotriptor under direct vision through suprapubic catheter tract (10).

We faced management dilemma in the current case. The placement of the urethral catheter was contraindicated due to blocked, encrusted SPC and underlying urethral injury. The treatment of encrustation with ESWL was not suitable because of warfarin use and possible risk of bleeding during the procedure. The use of the lithotripter requires repeated insertion through urethral that can worsen the urethral injury. Therefore, we adopted a safe and quick procedure to remove encrusted SPC that did not jeopardise the catheter track and patient's safety.

CONCLUSION

Encrusted catheter is a common intricacy in urology practice that can be treated in several ways. To the best of our knowledge, we reported the first case of treatment of encrustation with artery forceps by using 5 mm 0° cystoscope in high-risk patient with pulmonary embolism and urethral injury, where conventional methods of treatment are contraindicated.

LEARNING POINTS

The current report is a rare case of SPC encrustation complicated by urethral injury and pulmonary embolism.

ESWL and EL are conventional and gold standards to manage such patients in routine practice

Conventional treatments for encrustation were not suitable in our patient because of urethral injury and anticoagulants use.

We developed a safe and quick technique to break encrusted SPC without harming catheter track.

A new technique used to treat such high-risk patients will aid clinicians to manage such patients in a safe manner.

AUTHORS' NOTES

AAH and MNGR performed the procedure in the current patient. ASA and AHK carried out radiological and laboratory investigations. THM and YHK wrote the manuscript and approved the final draft.

REFERENCES

1. Jacob P, Rai BP, Todd AW. Suprapubic catheter insertion using an ultrasound-guided technique and literature review. BJU Int 2012; **110:** 779–84.
2. Perrin LC, Penfold C, McLeish A. A prospective randomized controlled trial comparing suprapubic with urethral catheterization in rectal surgery. Aust N Z J Surg 1997; **67:** 554–6.
3. Adeyemo B, Makovitch S, Foo D. A peculiar complication of suprapubic catheterization: recurrent ureteral obstruction and hydronephrosis. J Spinal Cord M 2013; **36:** 166–9.
4. Olapade-Olaopa EO, Adebayo SA, Atalabi OM, Popoola AA, Ogunmodede IA, Enabulele UF. Rigid retrograde endoscopy under regional anaesthesia: a novel technique for the early realignment of traumatic posterior urethral disruption. Afr J Med Med Sci 2002; **31:** 227–80.
5. Gonzalgo ML, Patrick CW. Balloon cuffing and management of the entrapped Foley catheter. Urology 2003; **61:** 825–7.
6. Hedelin H, Eddeland A, Larsson L, Pettersson S, Ohman S. The composition of catheter encrustations, including the effects of allopurinol treatment. Br J Urol 1984; **56:** 250–4.
7. Mobley HL, Warren JW. Urease-positive bacteriuria and obstruction of long-term urinary catheters. J Clin Microbiol 1987; **25:** 2216–7.
8. Canby-Hagino ED, Caballero RD, Harmon WJ. Intraluminal pneumatic lithotripsy for the removal of encrusted urinary catheters. J Urol 1999; **162:** 2058–60.

9. Kunzman SA, Srinadh ES, Lala SM, Albusaidi Q. Management of retained encrusted urethral catheter with extracorporeal shockwave lithotripsy. Indian J Urol 2002; **19:** 83–4.
10. Christopher CK Ho, Yugasaravanan K, Praveen S, Eng Hong G, Zulkifli Z. Encrusted and incarcerated urinary bladder catheter: what are the options? Libyan J Med 2010; **5:** 1–3.

CASE REPORT

A Painful S-100 (−)/CD68 (+) Myxoid-hypocellular Neurothekeoma: A Case Review through an Unusual Nerve Sheath Tumour

B Tas[1], S Altinay[2], HN Azakli[1]

ABSTRACT

Neurothekeomas are very rare, benign, soft-tissue tumours that originate from the sheath of the peripheral nerves. They are commonly located on the upper extremities or the head and the neck. Pain is an unusual, clinical symptom. We report a case of a 69-year-old woman, who presented to us with a painful nodule on the upper inner surface of the left leg. With the histopathological, histochemical and immunostaining features, the lesion was diagnosed as S-100 (−)/CD68 (+) myxoid hypocellular neurothekeoma. The case was presented due to the unusual clinicopathological features.

Keywords: Leg, myxoma, nerve sheath tumours, neurothekeoma, painful.

INTRODUCTION

Neurothekeoma is an uncommon, benign, soft-tissue tumour that originates from the sheath of the peripheral nerves with fairly distinctive histological features (1). It is commonly located on the head, neck and upper extremities of young females in the second or third decades of life (2, 3). The tumours are mainly classified into three groups according to cellularity of the tumour, growth pattern, and amount of stromal mucin as myxoid, cellular, and mixed type (2, 4). Immunohistochemical staining features can help to distinguish the histological variants of the tumour, and also differentiate it from other nervous tissue tumours and melanocytic tumours (1–3). Due to the rarity of neurothekeomas, lack of specific clinical and histological features, and non-standardised immunohistochemical markers for identification, there is no consensus about certain diagnostic criteria, and they are increasingly subjected to conceptual changes in classification (1, 2, 4).

CASE REPORT

A 69-year-old woman was admitted to our dermatology clinic with a tender protrusion located on her left leg which was present for 6 years without any history of trauma and precursor lesion. There was no additional complaint or other significant medical or family history. The dermatological examination showed a pinkish-purple, semi-translucent, moderately hard, elastic in consistency, 4.5 × 2.5 cm in size, solid and fusiform soft-tissue tumour on the upper inner surface of the left leg (Fig. 1A, 1B). The patient stated that she did not sleep and walk easily due to the pricking pain, especially with lateral pressure on the lesion.

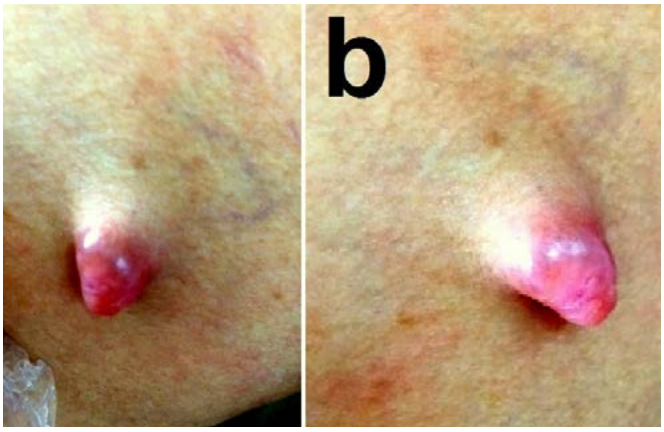

Fig. 1: Clinical views of the lesion: (A) distant, (B) closer.

Routine laboratory examinations of the patient including a red blood cell count, haemoglobin, haematocrit, erythrocyte sedimentation rate, serum biochemistry and urinalysis were within normal limits. Physical examination did not reveal signs of regional or systemic

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lymphadenopathy or hepatosplenomegaly. Neurological examination was normal. Requested soft-tissue ultrasonography showed a well-vascularised, semi-hypoechoic, well-circumscribed dermal tumoural mass. Subsequently, the lesion was totally excised and the defect was closed primarily. Histopathological examination revealed an encapsulated, concentric, dermal tumour composed of a single large nodule with scattered stellate and spindle-shaped cells on a background of myxoid-rich eosinophilic stroma, and prominent angioplasia of the dermal vessels.

Cellularity of the tumour was considered to be very low (Fig. 2A–2C). It was filling the dermis and extended to subcutaneous fatty tissue but did not invade it. No vascular or perineural invasion, high mitosis or cytological pleomorphism were found. Histochemical staining demonstrated that the tumour cells were strongly positive for alcian blue (> 90%) (Fig. 2D). Immunohistochemical analyses of the cells were negative for S-100 (Fig. 2E, 2F) and glial fibrillary acidic protein (GFAP) (Fig. 2G), and were positive for CD68 (Fig. 2H) and vimentin (Fig. 2I). Additionally, there was focal and non-specific positivity for CD57 (Fig. 2J). Based on the histopathological and immunohistochemical findings, the lesion was diagnosed as S-100 (−)/CD68 (+) myxoid-hypocellular neurothekeoma. The patient was followed up for 15 months without evidence of recurrence.

DISCUSSION

Neurothekeoma was first described by Hakin and Reed in 1969, as nerve sheath myxoma, but the name 'neurothekeoma' was given by Gallager and Helwing in 1980 (5). The origin of these tumours has not been elucidated and their aetiology has been debated (2). However, they were considered to be derived from nerve cells or Schwann cells (1). The tumour is commonly presents as small, solitary, erythematous nodules on the face and the upper limb.

Other locations such as the oral cavity, *cauda equina*, lower limb, shoulder, and neck have been reported in literature (3). It can originate in mucosa or submucosa (6). The tumours are difficult to diagnose prior to performing a biopsy due to the lack of specific clinical manifestations or imaging characteristics (2). However, they are usually slow-growing, non-tender lesions, which are either dermal or subdermal (3, 4). Myxoid type is primarily found in adults, with a predilection for the head and neck region in women. The cellular variant was first described in 1986 by Rosati *et al.* stating that they were frequently observed in the face of young persons (1).

In our case, the lesion was painful and located on an unusual anatomic location of the upper leg and these unusual clinical features were not consistent with the common literature. Neurotehekeomas show three histological variants: myxoid (classical or hypocellular), cellular, and mixed (4), primarily on the basis of the amount of myxoid matrix (1, 4), cellularity, growth pattern (4), and S-100 positivity (7). Fetsch *et al.* classified as follows: tumours with > 50% myxoid matrix as myxoid type, tumours with > 10% but ≤ 50% myxoid matrix as mixed-type, and those with ≤ 10% myxoid matrix as cellular type (1). The histopathological findings of myxoid neurothekeomas are lobular to plexiform dermal tumour masses composed of spindle-to stellate-shaped cells, with a striking myxoid stroma. The cellular variants have an ill-defined fascicular growth pattern consisting of plump epithelioid spindle cells (8), and the

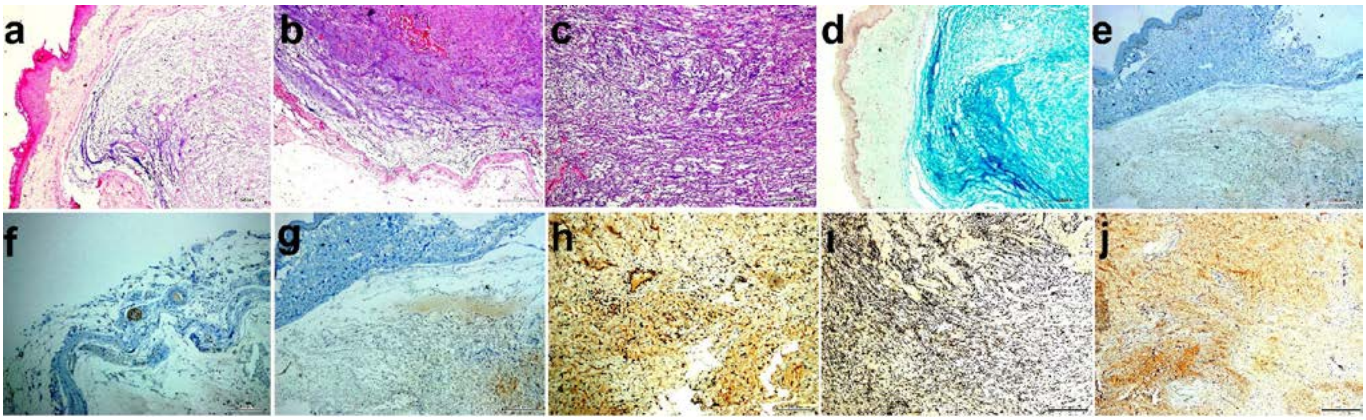

Fig. 2: Histopathological views of the lesion: (A) HE ×40, (B, C) HE ×100, (D) alcian blue ×40, (E) S-100 ×40, (F) S-100 positive control ×100, (G) GFAP ×40, (H) CD68 ×100, (I) Vimentin ×40, (J) CD57 ×40.

mucinous matrix is sparse or absent (1). They can show variable cytological atypia and mitotic activity (2, 7). The mixed type shares features of both (4).

Additionally, atypical cellular subtype of cellular neurothekeoma is characterised by some features such as large size of up to 6 cm, penetration into subcutaneous fat and/or muscle, diffusely infiltrating borders, vascular invasion, a high mitotic rate, and marked cytological pleomorphism (8). Our lesion did not show prominent cellularity, which was then considered as hypocellular (myxoid) type. It stained positively > 90% for alcian blue, which is a characteristic of the myxoid type. Additionally, the tumour was composed of a big, single nodule with delicate, concentric layers, and therefore it matched the lobular growth pattern that is attributed to the myxoid (hypocellular) type.

For these reasons, we first thought that our lesion could be a classical myxoid hypocellular neurothekeoma. On the other hand, it has been stated that the subtypes of neurothekeoma could be distinguished with some immunohistochemical markers such as S-100 protein, GFAP, nerve growth factor receptor (NGFR), CD57, NKI/C3, Ki-M1p, and CD68 (1). Myxoid types are typically positive for markers of nerve-origin cells, such as S-100 protein, GFAP, and NGFR, but not for macrophage markers including Ki-M1p and CD68. Therefore, myxoid subtypes are considered to be derived from nerve cells or Schwann cells (1). However, our lesion was negatively stained for S-100 and GFAP, but positive for CD68 which did not comply with the literature regarding the myxoid type. On the other hand, cellular type is usually negative for S-100, GFAP, NGFR, and CD57, but positive for NKI/C3, Ki-M1p and CD68. For this reason, it has been postulated that the cellular variant shows a fibrohistiocytic differentiation. Although S-100 negativity is known to be useful in differentiating the myxoid variants from the cellular ones, first Strumia *et al.* reported S-100 (−) myxoid neurothekeoma in 2001 (1,9).

Some authors suggested that myxoid neurothekeoma can lose the strong S-100B expression when cells are less differentiated (4). Only four S-100 (−) myxoid neurothekeomas have been reported in literature. However, in only one case, the fibrohistiocytic staining, such as CD68, was performed. In addition, the staining for vimentin is usually positive in both types (1) as seen in our case. However, it has been stated that fibrohistiocytic markers, such as vimentin, EMA, factor XIIIa or SMA, and neuroectodermal antigens, including neuron-specific enolase, NKI/C3, PGP 9.5, have restricted diagnostic value in determining the histological types. On the other hand, mixed types can have both types of features, but the immunostainings often exhibit non-regular features with an irregular or absent reactivity to S-100 protein and smooth muscle actin. Moreover, in 2002 Rudolph and Schubert first reported a 'myxoid cellular neurothekeoma' which has the typical histological features of the myxoid type, but was negative for S-100 protein or NGFR, and positive for NKI/C3 and Ki-M1p (1). In our case, although the lesion had high degree staining with alcian blue, low cellularity and lobular growth pattern which are specific for myxoid types, because of the negative staining for S 100 and GFAP and the positive staining for CD68 and vimentin, it was diagnosed as lobular, S-100 (−)/CD68 (+) myxoid-hypocellular neurothekeoma.

The cases of Rudolph and Schubert and Yun *et al.* (1) were similar to our lesion. However, although their lesions did not exhibit prominent cellularity, they named their lesions as 'myxoid cellular' according to only immunohistochemical staining features. However, we think that the term 'cellular' must only describe a histological feature rather than an immunohistochemical staining characteristic, and describing these complicated lesions according to only one diagnostic criterion can lead to diagnostic shortcomings. Therefore, we suggest that in the naming and classification of these tumours, the mucin content, growth pattern, cellularity and immunohistochemical staining features must be defined and evaluated separately to avoid diagnostic confusion.

In the main, differential diagnoses of neurothekeomas, fibroma, dermatofibroma, leiomyoma, neurilemmoma, neurofibroma (7), melanocytic tumours (5), skin cysts, or adnexal neoplasms (1) should be considered. The neurothekeomas are usually slow-growing, benign tumours, and if totally excised, do not recur. No metastasis has been reported (2, 4, 5). We think that elaborated clinical, histological, immunohistochemical and also electron-microscopic descriptions with the finest details of new cases will help us to better understand, and accurately diagnose these unusual and very rare tumours more easily. Therefore, reporting each different finding about new cases in the future is very important to avoid misdiagnosis, to make differential diagnosis easier, and to provide a diagnostic consensus. Our case was presented in order to emphasise the rarity, diagnostic difficulties and correct naming of neurothekeomas.

AUTHORS' NOTE

The authors declare that they have no conflict of interests.

REFERENCES

1. Yun SJ, Park HS, Lee JB, Kim SJ, Lee SC, Won YH. Myxoid cellular neurothekeoma: a new entity of S100-negative, CD68-positive myxoid neurothekeoma. Ann Dermatol 2014; **26**: 510–13.
2. Li J, Shi Z, Jing J. Neurothekeomas of the thoracic and lumbar area in an adult man: a case report. Mol Clin Oncol 2014; **2**: 156–8.
3. Stratton J, Billings SD. Cellular neurothekeoma: analysis of 37 cases emphasizing atypical histologic features. Mod Pathol 2014; **27**: 701–10.
4. Wollina U, Hansel G, Schönlebe J, Haroske G. Myxoid neurothekeoma—a painful case in a less common location. Dermatol Online J 2009; **15**: 3.
5. Akhtar K, Zaheer S, Ray PS, Sherwani RK. Myxoid neurothekeoma: a rare soft tissue tumor of hand in a male toddler. Niger J Surg 2013; **19**: 32–4.
6. Cribier B, Baran R, Varini JP. Nerve sheath myxoma of the hyponychium. Ann Dermatol Venereol 2013; **140**: 535–39.
7. Avninder S, Ramesh V, Vermani S. Benign nerve sheath myxoma (myxoid neurothekeoma) in the leg. Dermatol Online J 2007; **13**: 14.
8. Wilson AD, Rigby H, Orlando A. Atypical cellular neurothekeoma—a diagnosis to be aware of. J Plast Reconstr Aesthet Surg 2008; **61**: 186–8.
9. Strumìa R, Lombardi AR, Cavazzini L. S-100 negative myxoid neurothekeoma. Am J Dermatopathol 2001; **23**: 82–3.

CASE REPORT

Tigecycline-induced Hypofibrinogenaemia in a Patient with End-stage Renal Disease

S Wang, Z Li, L Li, F Liu

ABSTRACT

Tigecycline is a broad-spectrum antibiotic agent used to treat severe or multidrug-resistant (MDR) bacterial infections. Bacterial infection is a common and severe complication or comorbidity associated with end-stage renal disease (ESRD). However, few cases have been reported regarding the adverse drug reactions of tigecycline in patients with ESRD. Here, we detail the case of a 19-year-old female with ESRD, who received tigecycline to treat her sepsis due to an MDR Staphylococcus aureus*. Following 7 days of tigecycline, the patient developed coagulopathy with hypofibrinogenaemia, although there was no subsequent haemorrhage. The hypofibrinogenaemia resolved within 14 days after the discontinuation of tigecycline. Therefore, we recommend that clinicians strictly monitor the coagulation parameters in patients with ESRD during tigecycline treatment.*

Keywords: End-stage renal disease, hypofibrinogenaemia, tigecyceline.

INTRODUCTION

Tigecycline presents excellent reactions against a broad spectrum of bacteria including multidrug-resistant Gram-negative bacteria (1). Bacterial infection is a common and severe complication of end-stage renal disease (ESRD), for which the choice of antibiotics is difficult due to drug metabolism and renal insufficiency. Tigecycline represents an effective alternative, but concerns remain regarding the safety of tigecycline in patients with ESRD. Here, we report a case of an ESRD patient infected by *Staphylococcus aureus* who experienced hypofibrinogenaemia with the use of tigecycline.

CASE REPORT

A 19-year-old Tibetan girl presented with recurrent facial oedema and fatigue. She was diagnosed with chronic renal disease in Tibetan clinics and treated with traditional Tibetan medicine for 1 year. Two months before her admission, her oedema exacerbated, with cough, expectoration, dyspnoea, coma, recurrent high fever, dyskinesia in the right upper limb and epilepsy. The laboratory tests indicated that she was suffering from hypertension, severe anaemia, significant increase of serum creatinine (685 μmol/L) and white blood cell (WBC) count (19.3×10^9/L), pulmonary infections, a left cerebral infarction and encephalatrophy. She had been administered antibiotics (amoxicillin-sulbactam, imipenem and moxifloxacin), regular haemodialysis, invasive mechanical ventilation and other supportive treatments, but her symptoms did not disappear.

On her admission, the patient was conscious and her vital signs were acceptable. The laboratory tests revealed the following: WBC 15.48×10^9/L, haemoglobin 84 g/L, serum creatinine 286.0 μmol/L and albumin 25.3 g/L. Her sputum culture showed Gram-positive streptococcus. Blood from her peripheral vein and venous catheter in addition to urine were collected and sent for Gram stain microbiological culture.

This patient urgently needed a powerful antibiotic agent and broad-spectrum antibiotic coverage. Because the patient had prior exposure to many antibiotics, tigecycline was selected as the antibiotic medicine. Tigecycline monotherapy (50 mg q12 h iv) was initiated, and its efficacy was later confirmed by the isolation of tigecycline-sensitive *Staphylococcus aureus* from her blood culture and a sensitive *Escherichia coli* from her urine culture. New catheters were inserted, and regular haemodialysis and other supportive treatments were administered.

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After the start of tigecycline therapy, symptoms of fever, cough and expectoration gradually disappeared, and no spasm occurred. The patient's WBC count (Fig. 1) and other inflammatory parameters (*eg*, PCT and CRP) were strictly monitored and improved dramatically. However, we observed hypofibrinogenaemia (1.73 g/L), and worsening of prothrombin time (PT) and activated partial thromboplastin time (APTT) on day 7 of the tigecycline treatment (Fig. 2). As no signs of haemorrhage or disseminated intravascular coagulation (DIC) were observed, we did not change the treatment strategy. On day 11, her fibrinogen decreased sharply to an intolerable 0.86 g/L, with a slight declination in PT and fluctuation of APTT (Fig. 2B). At this point, tigecycline was discontinued, as it was suspected to be responsible for this coagulation disorder. Moxifloxacin and vancomycin were selected as antibiotics after tigecycline withdrawal according to the drug sensitivity tests. Meanwhile, we administered fresh-frozen plasma transfusions to protect the patient from bleeding.

From that day onward, her fibrinogen climbed slowly and steadily to reach the lower limit of normal, and PT and APTT stayed within the normal range. Fourteen days after the discontinuation of tigecycline, her fibrinogen improved and remained within the normal level without the administration of fresh-frozen plasma transfusions (Fig. 2A). Repeated blood, urine and sputum cultures found no evidence of bacterial growth. The patient recovered and remained stable without any recurrence of fever, cough, epilepsy or coagulation disorder. The patient is now waiting for a kidney transplant.

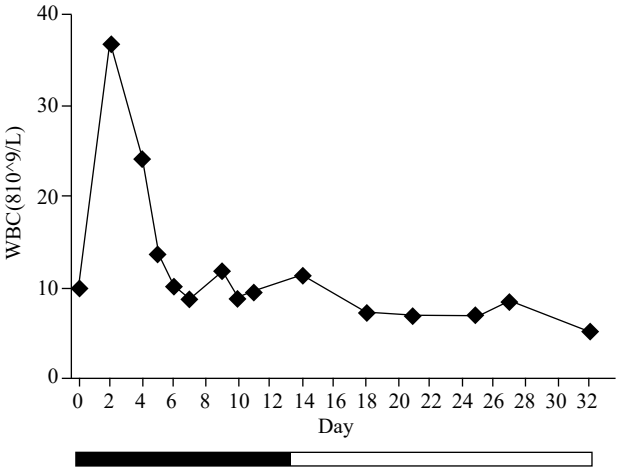

Fig. 1: Trend in WBC level. A gradual decrease in WBC counts with tigecycline administration (black bar) and other antibiotics following withdrawal of tigecycline (white bar). WBC = white blood cell.

DISCUSSION

Tigecycline is a relatively novel antibiotic agent with powerful antibiotic reactions against a wide spectrum of bacterial organisms. Tigecycline is mainly metabolised by the liver, and it requires no dosage adjustment in patients with renal impairment (2), making it a good alternative for infections in patients with chronic renal disease or ESRD. In our case, an ESRD patient presented with sepsis, severe and complicated bacterial infection, and high risk of persisting infections, as

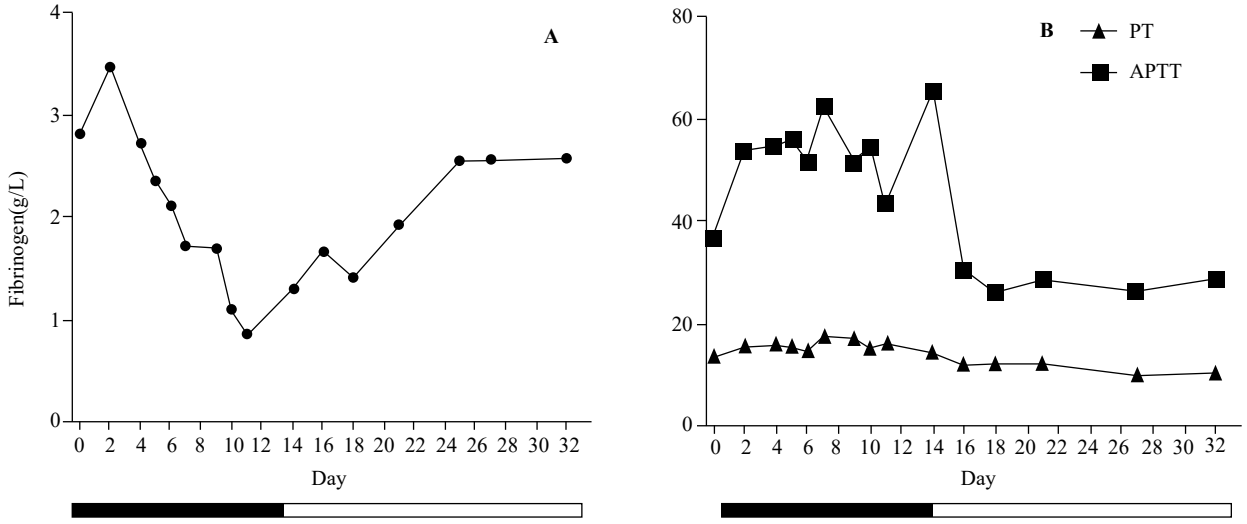

Fig. 2: Trends in fibrinogen level and PT/APTT with (black bar) or without (white bar) use of tigecycline. (A) A remarkable decrease in fibrinogen on day 7–11 of tigecycline administration, resolved gradually by its withdrawal. (B) Fluctuation of PT and APTT with the use of tigecycline and improved by its withdrawal. PT = prothrombin time; APTT = activated partial thromboplastin time.

well as resistance to numerous antibiotics. Tigecycline resolved her symptoms and improved her parameters of infections. Hypofibrinogenaemia occurred after 7 days of tigecycline treatment and was relieved after tigecycline was discontinued, with no changes in other therapies. Sepsis or severe infection was the most likely cause of her hypofibrinogenaemia; however, systemic inflammation and the general condition of the patient were improving at onset. Other causes of hypofibrinogenaemia included congenital hypofibrinogenaemia, DIC, leukaemia, malignant tumour, severe liver diseases and hemophagocytic syndrome; nevertheless, the patient displayed no evidence of these diseases. Thus, we hypothesised that hypofibrinogenaemia was a side effect of tigecycline.

It is suggested that tigecycline causes nausea, vomiting, pancreatitis, hepatic injury or failure and hypoglycaemia with a relatively high frequency (3). However, few cases of hypofibrinogenaemia have been reported as an adverse drug reaction to tigecycline. Rossitto presented a case of tigecycline-induced coagulopathy and hypofibrinogenaemia in a patient with advanced liver cirrhosis (4). Pieringer reported a case in which the use of tigecycline was associated with hypofibrinogenaemia in an ESRD patient undergoing continuous ambulatory peritoneal dialysis (5). In addition to this case, this study further indicates that tigecycline is a probable cause of hypofibrinogenaemia in patients with ESRD.

Hypofibrinogenaemia is rarely found in ESRD patients. In contrast, these patients have increased levels of fibrinogen, which directly contributes to a hypercoagulable state (6, 7), thus increasing the risk of venous thromboembolism (8, 9). However, coagulation disorders, which increase the risk of bleeding, are the common complications in ESRD patients; the most prominent abnormality is the prolongation of skin bleeding time (10), while APTT or PT is usually in the normal range. In this study, the fibrinogen level in the patient was initially normal, but it dropped with the use of tigecycline and recovered after the discontinuation of this drug. Therefore, we believe that tigecycline caused hypofibrinogenaemia in this ESRD patient.

Due to the increasing use of tigecycline for treating complicated infections, more attention should be paid to the adverse drug reactions associated with this drug. In conclusion, we strongly recommend the strict monitoring of fibrinogen and other coagulation parameters in patients who are receiving tigecycline as an antibiotic agent.

ACKNOWLEDGEMENTS

Many thanks to the patient, her family, and all the medical and paramedical staff of West China Hospital of Sichuan University.

REFERENCES

1. Frampton JE, Curran MP. Tigecycline (vol 65, p. 2631, 2005). Drugs 2006; **66:** 448.
2. Korth-Bradley JM, Troy SM, Matschke K, Muralidharan G, Fruncillo RJ, Speth JL et al. Tigecycline pharmacokinetics in subjects with various degrees of renal function. J Clin Pharmacol 2012; **52:** 1379–87.
3. Kadoyama K, Sakaeda T, Tamon A, Okuno Y. Adverse event profile of tigecycline: data mining of the public version of the US Food and Drug Administration adverse event reporting system. Biol Pharm Bull 2012; **35:** 967–70.
4. Rossitto G, Piano S, Rosi S, Simioni P, Angeli P. Life-threatening coagulopathy and hypofibrinogenaemia induced by tigecycline in a patient with advanced liver cirrhosis. Eur J Gastroen Hepat 2014; **26:** 681–4.
5. Pieringer H, Schmekal B, Biesenbach G, Pohanka E. Severe coagulation disorder with hypofibrinogenemia associated with the use of tigecycline. Ann Hematol 2010; **89:** 1063–4.
6. Shlipak MG, Fried LF, Crump C, Bleyer AJ, Manolio TA, Tracy RP et al. Elevations of inflammatory and procoagulant biomarkers in elderly persons with renal insufficiency. Circulation 2003; **107:** 87–92.
7. de la Serna G. Fibrinogen: a new major risk factor for cardiovascular disease. A review of the literature. J Fam Pract 1994; **39:** 468–77.
8. Parikh AM, Spencer FA, Lessard D, Emery C, Baylin A, Linkletter C et al. Venous thromboembolism in patients with reduced estimated GFR: a population-based perspective. Am J Kidney Dis 2011; **58:** 746–55.
9. Pavord S, Myers B. Bleeding and thrombotic complications of kidney disease. Blood Rev 2011; **25:** 271–8.
10. Boccardo P, Remuzzi R, Galbusera M. Platelet dysfunction in renal failure. Semin Thromb Hemost 2004; **30:** 579–89.

LETTER

A Case of Glomangiopericytoma of the Nasal Septum

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The Editor,

Sir,

A 78-year-old female patient presented to our clinic with congestion in the right nasal cavity and recurrent epistaxis for a month period. The medical history was not significant except hypertension, hyperlipidemia and rheumatoid arthritis. On physical examination, there was a 15 mm diameter, painless and bleeding tumour at the right side of the nasal cavity (Fig. 1A).

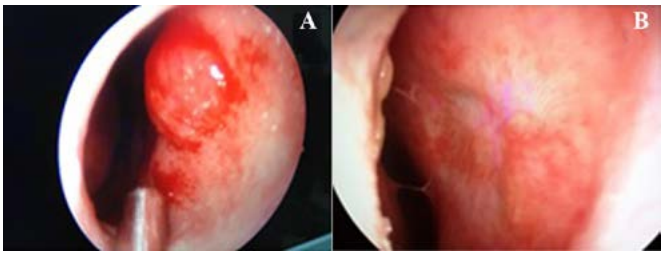

Fig. 1 (A) Right nasal endoscopy showing a haemorrhagic mass. (B) The 15 months of patient follow-up, no signs of recurrence were observed.

Endoscopic examination revealed a haemorrhagic mass in the right nasal septum. At labarotory evaluation there were no abnormal results. The haemorrhagic mass of the septum was widely excised endoscopically including the septum mucosa and the perichondrium. In immunohistochemical assessment, Vimentin was (+), SMA (+) and CD31 (+); CD34 focal positive; Desmin (−), S-100 (−), HMB-45 (−) and CK (−) and Ki-67 index was under 1% (Fig. 2A–E).

The tumour was histopathologically diagnosed as glomangiopericytoma. During the 15 months of patient follow-up, no signs of recurrence were observed (Fig. 1B).

Glomangiopericytoma, which is a type of sinonasal hemangiopericytoma, arises from the pericytes surrounding capillaries. Glomangiopericytoma is a very rare type of nasal cavity tumour, which shows a very slight rise in the female sex, and incidence age peak is during the seventh decade of life and varies in malignancy potential (1, 2). This tumour was classified as glomangiopericytoma by the World Health Organization (WHO) in 2005 (3). Glomangiopericytoma is described in WHO classification as a borderline low malignancy tumour. The local recurrence rate is 16.8%. However, recurrence can occur as a result of incomplete excision. Thus recurrence is better considered as residual disease in such cases (4). Differential diagnosis includes similar pattern soft tissue tumours such as a solitary fibrous tumour, sinovial sarcoma, hemangioendotelioma, glomus tumour and conventional haemangiopericytomas (5).

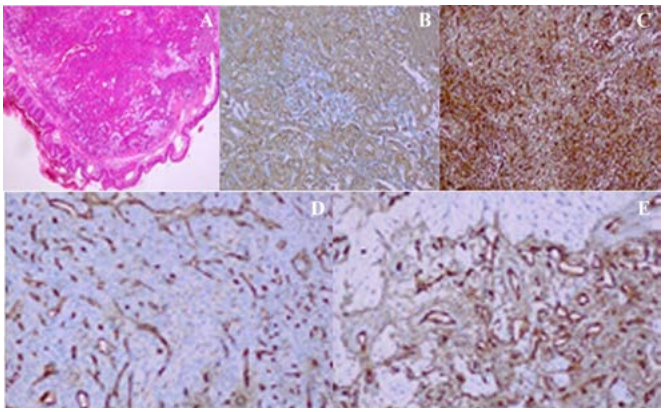

Fig. 2: (A) Hematoxylin-eosin staining reveals nodular lesion which is seperated from the squamous metaplastic epithelium of the respiratory tract with a clear neat zone. (original magnification ×10). (B) The results of immunohistochemical staining SMA (+) in the lining cells of the lesion (original magnification ×40). (C) The results of immunohistochemical staining Vimentin (+) in the cells that make up the lesion. (original magnification ×40). (D) The results of immunohistochemical staining CD 31 (+) in the vessel walls of the lesion (original magnification ×40). (E) The results of immunohistochemical staining CD 34 (+) in the vessel walls and peripheral cells of the lesion (original magnification ×40).

Malignancy in glomangiopericytomas is uncommon and malignant tumours display nuclear pleomorphism, high mitotic activity and necrosis (4). In our immunohistochemical assessment, Vimentin was (+), SMA (+) and CD31 (+); CD34 focal positive; Desmin (−), S-100 (−), HMB-45 (−) and CK (−) and Ki-67 index was under 1%. These results diagnosed the tumour as glomangiopericytoma. The gold standard treatment is usually accepted as resection with tumour-free margins (5). In our case, the tumour was widely excised including the septum mucosa and perichondrium. In our case, during the 15 months of patient follow-up, no signs of recurrence were observed.

Glomangiopericytoma is rarely detected at the nasal septum. Definitive diagnosis should be supported with clinical examination and histopathologic and immunohistochemical features. This rarely seen tumour should be kept in mind when evaluating nasal masses. Wide local excision is almost always sufficient for treatment but close follow-up is extremely important.

Keywords: Glomangiopericytoma, epistaxis, nasal mass, nasal septum.

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REFERENCES

1. Kanazawa T, Nishino H, Miyata M, Kuriki K, Abe K, Ichimura K. Haemangiopericytoma of infratemporal fossa. J Laryngol Otol 2001; **115:** 77–9.
2. Thompson LD. Sinonasal tract glomangiopericytoma (hemangiopericytoma). Ear Nose Throat J 2004; **83:** 807.
3. Barnes L, Eveson JW, Reichart P, Sidransky D, Eds. World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours. IARC Press: Lyon 2005.
4. Thompson L, Miettinen M, Wenig B. Sinonasal-type hemangiopericytoma: a clinicopathologic and immunophenotypic analysis of 104 cases showing perivascular myoid differentiation. Am J Surg Pathol 2003; **27:** 737–49.
5. Hanege FM, Uzun L, Yavuz C, Ozkanli S, Kurtgoz S. Epithelioid hemangioendothelioma of the nasal septum. B-ENT 2016; **12**: 155–7.

LETTER TO THE EDITOR

A Giant Pituitary Adenoma in a Patient under Salmon Calcitonin Treatment: Coincidence or a Priori Consequence

M Kara[1], Ö Kara[2], L Ozçakar[1]

The Editor,

Sir,

A 57-year-old woman with hypertension had been diagnosed with postmenopausal osteoporosis (OP), and nasal salmon calcitonin treatment had been started in June 1997. In October 1997, she had been admitted for headache and hypertension. Cranial computed tomography—performed for hypertension—had been normal, and she had been successfully treated with antihypertensive therapy. She had been regularly followed during salmon calcitonin therapy until 2002.

In November 2002, the patient presented with headache, ptosis, diplopia, and loss of vision. Magnetic resonance imaging demonstrated a heterogeneous lesion (4.1 × 3.6 × 3.7 cm) invading sphenoid/cavernous sinuses, eroding clivus and pituitary stalk and compressing the optic chiasm (Figure). Laboratory analysis for hormone levels was normal (Table). Overall, the patient was diagnosed as a non-functioning pituitary macroadenoma. First, she underwent transsphenoidal adenoma excision and 1 month later, right pteroinal craniotomy. As thyroid-stimulating hormone and growth hormone levels decreased postoperatively (Table), thyroid replacement therapy and alendronate (instead of calcitonin) was prescribed. Postoperative course was otherwise uneventful with partial improvement in the oculomotor palsy and the residual macroadenoma was stabilised (2.5 × 2.5 × 1.5 cm). One-year later, gamma knife radiosurgery was also performed. To date, she has been followed regularly without any additional neurologic/hormonal dysfunction.

Pituitary adenomas are rare benign tumours, classified according to the size as microadenomas < 1 cm or macroadenomas > 1 cm; or according to hormonal hypersecretion as functional or nonfunctional (1). Pituitary macroadenomas often cause neurological symptoms due to mass effects such as visual field disturbances and headache (2). Surgery is the first-line treatment, and gamma knife radiosurgery and radiotherapy are effective for residual tumours.

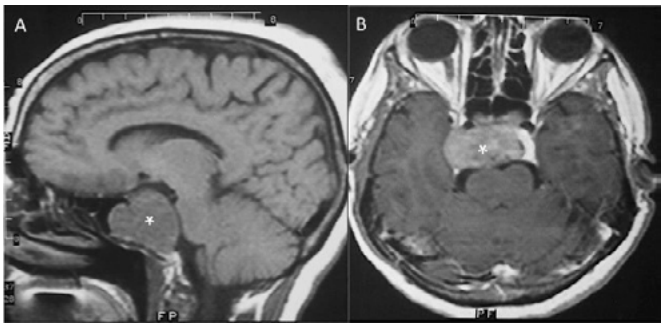

Figure: T1-weighted magnetic resonance imaging: (A) sagittal view and (B) axial view (with gadolinium enhancement) showing the pituitary macroadenoma with massive suprasellar extension and invasion to the sphenoid and cavernous sinuses mainly on the right side.

Table: Serum pituitary hormone levels of the patient in different times

	Before surgery	After surgery	Current
TSH (0.27–4.2 μIU/mL)	2.11	0.11	2.48
GH (1–9 ng/mL)	2.6	0.19	1.1
ACTH (9–52 pg/mL)	10.5	25.3	30.5
PRL (1.9–25 ng/mL)	14.5	7.39	9.2
FSH (25.8–134.8 μIU/mL)	33.56	39.02	36.1
LH (7.7–58.5 μIU/mL)	12.53	12.26	14.1

TSH = thyroid-stimulating hormone; GH = growth hormone; ACTH = adrenocorticotropic hormone; PRL = prolactin; FSH = follicle-stimulating hormone; LH = luteinizing hormone.

Calcitonin is mainly synthesised by C-cells of the thyroid gland and also secreted in several other neuroendocrine organs such as brain, lungs and pituitary gland (3). On the other hand, calcitonin receptors are mainly found on osteoclasts and at certain sites in the nervous system including hypothalamus, circumventricular organs, preoptic nucleus and anterior pituitary (4). Calcitonin inhibits osteoclastic activity leading to reduced bone resorption. Salmon calcitonin, a more potent form than human calcitonin, has been used for the treatment of OP without any major adverse effects. However, salmon calcitonin administration for 1 year was found to be associated with pituitary gland hyperplasia/adenoma in rats (5, 6). Although we were not able to exclude a sporadic pituitary microadenoma in our patient before the calcitonin treatment, we did not have any detailed histological/biochemical analysis of

the surgical specimens demonstrating the expression of the calcitonin receptor; we propose that calcitonin might have induced/accelerated the development of an adenoma.

Presenting the first case in the literature, we imply that screening studies are definitely awaited before we can conclude that our observation does not fall beyond a coincidence. Additionally, clinicians prescribing calcitonin should consider such a possibility and be vigilant against hormonal/neurological consequences due to aforementioned mechanisms.

Keywords: Calcitonin, diplopia, osteoporosis, pituitary adenoma.

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REFERENCES

1. Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML et al. The prevalence of pituitary adenomas. Cancer 2004; **101:** 613–9.
2. Colao A, Di Somma C, Pivonello R, Faggiano A, Lombardi G, Savastano S. Medical therapy for clinically non-functioning pituitary adenomas. Endocr Relat Cancer 2008; **15:** 905–15.
3. Sun YP, Lee TJ, Shah GV. Calcitonin expression in rat anterior pituitary gland is regulated by ovarian steroid hormones. Endocrinology 2002; **143:** 4056–64.
4. Vik A, Yatham LN. Calcitonin and bipolar disorder: a hypothesis revisited. J Psychiatry Neurosci 1998; **23:** 109–17.
5. Brown WR, Fetter AD, Van Ryzin RJ, Langloss JM. Proliferative pituitary lesions in rats treated with salmon or porcine calcitonin. Toxicol Pathol 1993; **21:** 81–6.
6. Ishii J, Katayama S, Itabashi A, Takahama M, Kawazu S. Salmon calcitonin induces pituitary tumor in rats. Endocrinol Jpn 1991; **38:** 705–9.

LETTER TO THE EDITOR

Rivaroxaban-Induced Protein S Deficiency

The Editor,

Sir,

Protein S is a vitamin K-dependent plasma glycoprotein with anticoagulant properties (1). It acts as a cofactor for activated protein C and tissue factor pathway inhibitor (TFPI). Tissue factor pathway inhibitor tightly binds to factor Xa and slowly inhibits it (2). Protein S deficiency can be hereditary, or acquired due to vitamin K deficiency, chronic liver disease, nephrotic syndrome, malignancy, systemic lupus erythematosus, disseminated intravascular coagulation, pregnancy, oestrogen use as well as recent surgery and thrombosis (3–6). Warfarin decreases protein S activity by inhibiting its vitamin K-dependent synthesis in liver (3). In this article, we report a patient with deep vein thrombosis who developed protein S deficiency on rivaroxaban therapy.

A 33-year-old male presented to the haematology clinic for the evaluation of a hypercoagulable state. The patient had been taking rivaroxaban for over a year after he was diagnosed with deep venous thromboses in the right brachiocephalic, subclavian and cephalic vein by Doppler ultrasonography. At the time of diagnosis of deep venous thrombosis, the patient was receiving total parenteral nutrition and antibiotics for gastroparesis and bacteraemia through an intravenous line in his right upper extremity. He had no family history of blood dyscrasias or primary hypercoagulable states. On examination, his vitals were normal. Systemic examination was negative for lymphadenopathy or hepatosplenomegaly. Laboratory investigations showed normal blood counts. His liver function and kidney function were normal. Hypercoagulability workup was negative for antiphospholipid antibodies, factor V mutation and G20210A mutation in the prothrombin gene. Further studies showed normal antithrombin 3 and protein C activity. However, protein S activity was 63% (reference range: 70%–150% normal). Repeat protein S activity after two months was still low at 63%. The patient's anticoagulation therapy was changed from rivaroxaban to low-molecular-weight heparin (enoxaparin). After two weeks of switching the medication, his protein S activity increased to 75%.

Rivaroxaban is a selective, competitive and direct inhibitor of factor Xa (7). It is an oral anticoagulant used for the treatment and prophylaxis of deep vein thrombosis and pulmonary embolism and for prevention of stroke and systemic embolism due to nonvalvular atrial fibrillation (8, 9). To the best of our knowledge, this is the first reported case of rivaroxaban-induced protein S deficiency as suggested by the temporal association between rivaroxaban and reduction in protein S activity. The exact mechanism is not clear. During the hypercoagulation workup, it may be important to switch rivaroxaban to unfractionated heparin or low-molecular-weight heparin prior to estimating protein S activity.

Keywords: Protein S, protein Xa, rivaroxaban

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REFERENCES

1. García de Frutos P, Fuentes-Prior P, Hurtado B, Sala N. Molecular basis of protein S deficiency. Thromb Haemost 2007; **98:** 543–56.
2. Comp PC, Nixon RR, Cooper MR, Esmon CT. Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest 1984; **74:** 2082–8.
3. Lipe B, Ornstein D. Deficiencies of natural anticoagulants, protein C, protein S, and antithrombin. Circulation 2011; **124:** e365–8.
4. D'Angelo A, Vigano-D'Angelo S, Esmon C, Comp P. Acquired deficiencies of protein S. Protein S activity during oral anticoagulation, in liver disease, and in disseminated intravascular coagulation. J Clin Invest 1988; **81:** 1445–54.
5. Van Ommen C, Fijnvandraat K, Vulsma T, Delemarre-van de Waal H, Peters M. Acquired protein S deficiency caused by estrogen treatment of tall stature. J Pediatr 1999; **135:** 477–81.
6. Song K, Park Y, Kim H. Prevalence of anti-protein S antibodies in patients with systemic lupus erythematosus. Arthritis Rheum 2000; **43:** 557.
7. Samama M. The mechanism of action of rivaroxaban—an oral, direct Factor Xa inhibitor—compared with other anticoagulants. Thromb Res 2011; **127:** 497–504.
8. Mueck W, Schwers S, Stampfuss J. Rivaroxaban and other novel oral anticoagulants: pharmacokinetics in healthy subjects, specific patient populations and relevance of coagulation monitoring. Thromb J 2013; **11:** 10.

9. Thomas T, Ganetsky V, Spinler S. Rivaroxaban: an oral factor Xa inhibitor. Clin Ther 2013; **35:** 4–27.