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Conference Proceedings: Bengisson S. Sothemin BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. pp.1561-5.

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study Kidney Int: 2004. Report No: 26.

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Review

Leishmaniasis in Northern Cyprus

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ABSTRACT

Leishmaniasis is a vector-borne disease that is caused by *Leishmania* parasites. Sandflies are the vectors, and dogs are the primary reservoir of *Leishmania* spp. Cutaneous leishmaniasis (CL) is the most common form of the disease, whereas visceral leishmaniasis (VL) is the most severe form and is generally fatal if left untreated. The disease is seen in 98 countries and distributed through three regions on five continents. The island of Cyprus is located in the eastern part of the Mediterranean region where leishmaniasis is endemic. The presence of sandflies, canine leishmaniasis (CanL), and human VL and CL cases has been documented in Northern and Southern Cyprus. CanL cases were found at various rates between 1.9% and 13.2% in Northern Cyprus. In 1990, Leishmanin skin test positivity was detected in Northern Cyprus, and *Leishmania infantum* was found to be the infecting agent. In 2016, three pediatric VL cases caused by *L. infantum* were reported in Northern Cyprus. More recently, in a study conducted in Kyrenia District, three seropositive individuals have been detected. In the study, seven individuals, including the seropositive persons, were found to have a history of CL. Consequently, these studies indicate the presence of leishmaniasis in Northern Cyprus. Therefore, vector and reservoir control programs should be implemented for prevention of the disease.

Keywords: Cutaneous leishmaniasis, *Leishmania donovani, Leishmania infantum*, Northern Cyprus, visceral leishmaniasis

INTRODUCTION

Leishmaniasis is a vector-borne disease that is caused by obligate intracellular protozoa belonging to *Leishmania* species. Vectors of *Leishmania* parasites are phlebotomine sandflies, whereas animals, primarily dogs, serve as a reservoir for parasites (1). In addition to dogs, mice, foxes, cats, and also humans can be a reservoir for leishmaniasis (2). Leishmaniasis is endemic in the tropical and subtropical regions, as well as the Mediterranean Basin (1). The disease is seen in 98 countries and distributed through three regions on five continents (3).

The disease has four clinical forms. These are visceral leishmaniasis (VL) (also known as kala-azar), post-kala-azar dermal leishmaniasis (PKDL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis. According to the World Health Organization, nearly 1.3 million new cases of leishmaniasis (300,000 visceral and 1 million cutaneous or mucocutaneous forms) occur annually (3).

Visceral leishmaniasis is the most severe form of leishmaniasis. It is generally fatal if it is left untreated. The disease is characterized by fever, weight loss, splenomegaly, hepatomegaly, and anemia (4). Approximately 90% of VL cases are reported from Bangladesh, Brazil, Ethiopia, India, Nepal, South Sudan, and Sudan. Annually, 20,000-50,000 people are estimated to die from VL (3). Occasionally, VL can develop into a cutaneous disease (PKDL) following treatment (3). In this case, macular, maculopapular, or nodular rash is present as skin lesions. Post-kala-azar dermal leishmaniasis is typically seen in Sudan and less frequently in other countries of eastern Africa and the Indian region. Owing to the high number of organisms in the lesions, these patients can be a reservoir for infection with sandflies and maintain transmission of VL (1).

The most common form of leishmaniasis is CL. The primary skin lesions are ulcers that leave scars and cause severe disfigurement. Approximately 95% of CL cases are reported in the Mediterranean Basin, the Middle East, Central Asia, and the region of the Americas (4). In mucocutaneous leishmaniasis, destructive lesions occur in the mucosa of the nose, mouth, and throat. Approximately 90% of cases are documented from Brazil, Peru, and Bolivia (4).

Leishmaniasis is an important concern for public health in the Eastern Mediterranean Region. Both CL and VL are endemic in this region (5). Owing to climate changes, the area covered by sandflies is expanding in the Mediterranean region, leading to the emergence of leishmaniasis in this region (6).

Cyprus is situated in the eastern part of the Mediterranean region between 34° and 35° northern latitudes and 32° and 34° eastern longitudes. The island has a Mediterranean climate, in which summers are generally hot and dry and winters are mild (7). In the island, canine leishmaniasis (CanL) was prevalent before 1945, and *Phlebotomus tobbi* was shown to be the vector of *Leishma-*

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nia infantum (8). The sandfly fauna consisting of *Phlebotomus* and *Sergentomyia* species in Cyprus was documented for the first time in the 1940s (7). However, the number of sandflies declined considerably due to the malaria eradication program implemented between 1940 and 1950. Additionally, the control program against echinococcosis between 1970 and 1975 resulted in a remarkable decrease in the number of dogs. For these reasons, the prevalence of CanL reduced substantially in Cyprus for more than two decades after these campaigns. However, discontinuation of these programs led to an increase in the number of sandflies and dogs, and as a result, CanL cases occurred again (8).

This review will provide an overview of the literature on sandfly vectors, CanL, and human leishmaniasis cases reported on the island, with a particular focus on Northern Cyprus.

CLINICAL AND RESEARCH CONSEQUENCES

Sandfly Studies in Northern Cyprus

Various sandfly species have been demonstrated in Northern Cyprus. In the study conducted by Demir et al. (7), a total of nine *Phlebotomus* and three *Sergentomyia* species were identified among sandflies collected from 20 different residential settings in Northern Cyprus. The localities with the greatest number of sandfly species diversity were found to be Lapithos (Lapta) and Leonarisso (Ziyamet) with 12 and 9 different species, respectively (Figure 1). The highest number of *Phlebotomus* species was noted to be *Phlebotomus galilaeus*, which was followed by *Phlebotomus papatasi* and *P. tobbi* (7).

Töz et al. (9) collected sandflies from different localities in Kyrenia and Lapithos and detected six *Phlebotomus* and three *Sergentomyia* species. Among *Phlebotomus* species, *P. tobbi* was found at the highest rate, followed by *P. papatasi* and *P. galilaeus*. The authors did not detect any *Leishmania* promastigotes in sandflies (9). Notably, Ergunay et al. (10) identified *L. infantum* in eight pools of *P. tobbi* in Northern Cyprus. Among the positive pools of *P. tobbi*, three were obtained from Lapithos, whereas five were collected from Panagra (Geçitköy), a locality close to Lapithos (10). These residential settings are indicated in Figure 1.

CanL Cases in Northern Cyprus

Owing to the increased number of sandflies and dogs, a number of studies have been conducted to investigate CanL cases. In 2004, 3 (3.61%) of 83 dogs were found to be positive for CanL in Klepini (Arapköy), a village in the Kyrenia District. Of the 83 dogs, 2 were found to be positive by indirect fluorescent antibody test (IFAT) and rK39 test, and 1 was found to be positive by polymerase chain reaction (PCR) only. Additionally, borderline positive results were obtained in 13 (15.66%) dogs by IFAT. In the same study, three of five clinically suspected dogs were detected to be positive for CanL by PCR in 2012 (9).

In 2016, seropositivity in dogs was found to be 1.9% (n=2/105), whereas 1 (0.95%) dog was noted to be borderline positive by IFAT in Northern Cyprus. The seropositive dogs were noted to be from Kyrenia (n=1) and Famagusta (Magosa) (n=1), whereas the dog that was found to be borderline positive (n=1) was from Nicosia (Lefkoşa) (11). Another study published in the same year found CanL seropositivity to be 3.55% (n=10/281) by IFAT in Northern Cyprus. Rates of seropositivity were 1.72% (n=1/58) in Trikomo (İskele), 13.20% (n=7/53) in Kyrenia, 1.67% (n=1/60) in Famagusta, and 3.33% (n=1/30) in Morphou (Güzelyurt). The study found no CanL seropositivity in Nicosia (12).

Human Leishmaniasis Cases in Northern Cyprus

In Cyprus, human leishmaniasis was not investigated comprehensively in the past, and the presence of two infantile VL cases has been reported in humans since 1935 (8). Thereafter, only a few studies have investigated the presence of leishmaniasis. In 1990, Leishmanin skin test positivity was detected to be 10% in Kyrenia and 35% in Lapithos in Northern Cyprus. The causative agent was found to be *L. infantum* by DNA hybridization (13).

More than two decades later, in 2016, three pediatric VL cases were reported from Northern Cyprus. The first patient was a 16-month-old female from Vuno (Taşkent) in Kyrenia District. The second and third cases, both from Aytrias (Sipahi) in the Karpasia Peninsula, were 24-month-old female and 34-month-old male patients, respectively. All of three cases were detected to be positive by IFAT (1≥1/128), direct agglutination test (DAT; ≥1/400), and rK39 test. *Leishmania* amastigotes were identified in Giemsa-stained slides of each bone marrow aspirate. Furthermore, PCR and restriction fragment length polymorphism analyzes of the aspirates indicated that the causative agent was *L. infantum* in three VL cases. Patients were successfully treated with liposomal amphotericin B (14).

Figure 1. Map of Cyprus indicating the localities of reported visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and canine leishmaniasis (CanL) cases; sandflies (only in particular settings) in Northern Cyprus. Positive Leishmanin skin test results: Kyrenia (K) and Lapithos (La). Three pediatric VL cases: Vuno (V) and Aytrias (A). CL in a traveler: Lapithos (La). Seven past CL cases, including three seropositive individuals: Kyrenia District. Seropositivity in dogs: Kyrenia (K), Klepini (Kl), Trikomo (T), Famagusta (F), and Morphou (M); borderline positive result in Nicosia. Highest number of sandfly species diversity: Lapithos (La) and Leonarisso (Le). *Leishmania infantum* (+) in *Phlebotomus tobbi*: Lapithos (La) and Panagra (Pa). Polis (CL case in a traveler) and Paphos (a family cluster of four CL cases) located in the Greek Cypriot territory are also indicated in the figure. (The map was adapted from: http://www.worldatlas.com/webimage/countrys/europe/outline/cy.htm)

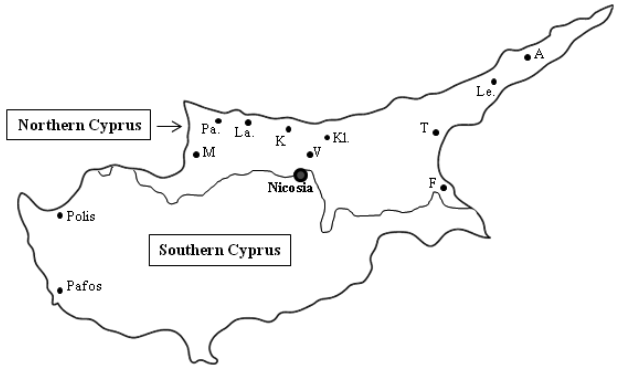

Table 1. Summary of reported human and canine leishmaniasis cases in Northern and Southern Cyprus in chronological order

	Northern Cyprus			Southern Cyprus		
Case	**Case definition**	**Reference**	**Ref. no.**	**Case definition**	**Reference**	**Ref. no.**
Human leishmaniasis	Positive Leishmanin skin test results in Kyrenia and Lapithos (1990). *L. infantum* is the causative agent.	Deplazes et al.	(13)	Three CL cases caused by *L. donovani* MON–37 (2006).	Antoniou et al.	(16)
	CL related with travel to Northern Cyprus. The causative agent is *L. donovani* complex.	de Silva et al.	(19)	Two VL cases caused by *L. infantum* MON–1 (2006).	Antoniou et al.	(16)
	Three pediatric VL cases caused by *L. infantum*.	Sayili et al.	(14)	CL associated with travel to Southern Cyprus. The causative agent is *L. donovani*/infantum complex.	Poeppl et al.	(18)
	Three (1.2%) seropositive individuals and 7 (2.8%) past CL cases (including the seropositive individuals) among 249 participants.	Ruh et al.	(15)	A family cluster of four CL cases caused by *L. donovani*.	Koliou et al.	(17)
Canine leishmaniasis	Three (3.61%) positive and 13 (15.66%) borderline positive results among 83 dogs (2004).	Töz et al.	(9)	CanL cases caused by *L. infantum* MON–1. Overall CanL seroprevalence was found to be 1.7% (1996).	Deplazes et al.	(13)
	Three CanL positive cases among five clinically suspected dogs (2012).	Töz et al.	(9)	Co–infection in a dog with L. infantum MON–1 and *L. donovani* MON–37.	Antoniou et al.	(16)
	Two (1.9%) seropositive and 1 (0.95%) borderline positive results in 105 dogs.	Beyhan et al.	(11)	CanL cases caused by mostly *L. infantum* MON–1. Seroprevalence was found to be 14.9%.	Mazeris et al.	(8)
	Ten (3.55%) seropositive results in 281 dogs.	Çanakçı et al.	(12)			

CL: cutaneous leishmaniasis; VL: visceral leishmaniasis; CanL: canine leishmaniasis

More recently, the presence of VL was evaluated in the Kyrenia District of Northern Cyprus, where CanL and sandflies were detected previously. Among 249 study participants, 3 (1.2%) were found to be seropositive, whereas the remaining 246 were found to be negative by serological tests. Two out of three participants (68- and 54-year-old males) were detected to be positive by DAT, and their serum antibody titers were 1:1600. The third case (18-year-old male) was detected to be positive by rK39 test. Furthermore, 7 (2.8%) of 249 participants, including the seropositive cases, had a history of CL (15).

The localities where human and CanL cases were reported in Northern Cyprus are indicated in Figure 1.

Studies from Southern Cyprus

The presence of sandflies, CanL, and human leishmaniasis in Southern Cyprus (Greek Cypriot territory) was also demonstrated by several studies. In 1996, CanL cases caused by *L. infantum* MON-1, the predominant zymodeme in the Mediterranean region, were detected in Southern Cyprus. In that study, the overall seroprevalence of CanL was found to be 1.7% (13). A later study reported the seroprevalence of CanL as 14.9% in Southern Cyprus. The parasite isolates collected from dogs were identified as *L. infantum* MON-1 and *L. infantum* MON-98 with rates of 98.4% and 1.6%, respectively. Additionally, in the study, *P. tobbi*, *P. galilaeus*, and *P. papatasi* were documented to be prevalent among *Phlebotomus* species collected (8).

In 2006, three CL (44-, 50-, and 55-year-old) and two VL cases (9-month-old and 73-year-old) were detected in humans in Southern Cyprus. These isolates were defined as *Leishmania donovani* MON-37. Additionally, in a dog living in the region where the three human CL cases were found, *L. infantum* MON-1 and *L. donovani* MON-37 were detected. This was interpreted in terms of a co-infection with two parasite species in the dog (16). In a village of the Paphos District in Southern Cyprus (Figure 1), a familial cluster of four CL cases developed by *L. donovani* was documented in 2014. Cases included a 6-year-old male patient,

a 60-year-old female patient, a 60-year-old male patient, and a 40-year-old female patient, respectively. The lesion in the third case self-resolved, whereas the other patients were administered liposomal amphotericin B (17).

Leishmaniasis Cases Associated with Travel to Cyprus

In the literature, there are also reports of CL cases that are related to travel to Cyprus. One of the patients (59-year-old female) had a history of a 2-week stay in Polis in the Greek Cypriot territory (Figure 1) 8 months before the diagnosis. The causative agent was identified as *L. donovani*/*infantum* complex, and the patient was successfully treated with miltefosine (18). Another case of CL (54-year-old male) spent 3 days in Lapithos in Northern Cyprus (Figure 1) 6 months before the onset of disease. The causative agent was detected to be *L. donovani* complex, and the patient was successfully treated with sodium stibogluconate (19).

Reported cases of human leishmaniasis and CanL in Northern and Southern Cyprus are summarized in Table 1.

Possible Transmission Cycles in Northern Cyprus

Previously, it was postulated that there are two cycles of leishmaniasis transmission in Cyprus. One of them involves *L. infantum* that causes CanL, and the second transmission is anthroponotic where *L. donovani* causes CL and VL in humans (17). The findings obtained in Southern Cyprus are consistent with this (8, 13, 16, 17). However, the types of transmission cycles in Northern Cyprus are yet to be cleared, as there are limited data on the molecular characteristics of *Leishmania* species infecting humans and dogs. Zoonotic transmission of *L. infantum* to humans is likely to occur, since *P. tobbi* (7) and CanL (9, 11, 12) have been demonstrated in different localities in Northern Cyprus. Nevertheless, considering that *P. tobbi* can be the vector for both *L. infantum* and *L. donovani* (8), the possibility of *L. donovani* transmission in humans cannot be ignored in Northern Cyprus. This hypothesis is strengthened by two facts. First, *L. donovani* is present in Southern Cyprus (16, 17). Second, a close genetic relationship was found between *L. donovani* isolates in Southern Cyprus and the strains in Turkey. It was also suggested that Turkey might be the origin of the Cypriot isolates (17, 20). Therefore, detailed analyzes should be conducted to better understand the molecular characteristics of *Leishmania* species in Northern Cyprus.

CONCLUSION

Previous and recent studies on leishmaniasis indicate that the disease is present in both humans and dogs in Cyprus (8, 9, 11-17). In Southern Cyprus, *L. donovani* MON-37 was found to be the primary zymodeme responsible for both CL and VL cases in humans (16). While a dog was found to be co-infected with both *L. donovani* MON-37 and *L. infantum* MON-1 (16), the major cause of CanL cases was detected to be *L. infantum* MON-1 in Southern Cyprus (8, 13). Studies from Northern Cyprus did not identify the detailed molecular characteristics of CanL cases (9, 11, 12), whereas *L. infantum* was reported as the infecting agent in human cases in this territory (13, 14). Moreover, *L. donovani* complex was shown to be responsible for a CL case in a traveler who visited Northern Cyprus (19).

Consequently, results obtained from the studies highlight that leishmaniasis is of concern not only in the south but also in the north of the island. Moreover, the presence of sandflies (7, 9, 10) and CanL cases (9, 11, 12) suggests that vector and reservoir control programs should be implemented in Northern Cyprus. In addition to the preventive measures, more studies are essential to investigate leishmaniasis in additional localities of the Turkish Cypriot territory. Furthermore, detailed molecular characteristics of *Leishmania* species should be identified in Northern Cyprus to evaluate their relationship with the parasites in Southern Cyprus and the neighboring countries.

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Review

Interactions between Parasites and Human Microbiota

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ABSTRACT

Parasitic infections are threatening millions of people, particularly in developing countries. These infections are usually associated with significant variability in clinical presentation from asymptomatic infection to chronic disease. It is suggested that the intestinal microbiota may help to explain the differences in disease expression. Recent studies reported that microbiota effect to parasite colonization and persistence in the host and the presence of parasitic infection may also alter the intestinal bacterial community. Microbiota plays an important role in protecting against pathogens and maintaining immune and metabolic homeostasis. The alteration of microbiota composition (dysbiosis) has been associated with the pathogenesis of many infections and inflammatory diseases. Understanding the interactions among microbiota, human parasites, and the host immune system may allow us for designing new treatment options for parasitic infections. The objective of this review was to summarize the recent development in this field.

Keywords: Helminth, intestinal microbiota, parasite, protozoa

INTRODUCTION

Humans are colonized by a variety of bacteria, fungi, viruses, and eukaryotic parasites in their intestines, mucosae, and skins. The term microbiota represents an ecological community of commensal microbes that live within the human body, and the term microbiome represents a total genome of the microbiota (1). Our gut microbiota contains approximately 10^{12} organisms/g at least 1000 different species of bacteria with nearly 3 million genes that are 150 times larger than human genes (1). Microbiota compositions can vary from one person to another (2). Human microbiota studies suggested that healthy or asymptomatic conditions areassociated with increased microbial diversity, and disease states are often linked to decreased bacterial diversity (3).

Microbiota is also well known for its role in the development and education of the immune system. These microbes not only modulate immune defense but also provide a variety of metabolic impact that usually is unfavor of colonization and invasion of pathogens. However, its interactions with diseases are still not well known. Environmental factors, such as diet, antibiotic usage, and lifestyle,may cause dysbiosis that is frequently associated with increased susceptibility to infections and non-infectious diseases (obesity, diabetes, allergy, and autoimmune and inflammatory diseases) (1, 3).

Globally, diarrhea is currently the second leading cause of death in children, and a large proportion of cases are caused by parasitic protozoans and helminths (4). *Entamoeba*, *Cryptosporidium*, and *Giardia* caused 357 million cases and resulted in almost 34,000 deaths annually (5). Malaria kills approximately 660,000 people/year, and most of them are young children under the age of five years (6). Recent estimates indicate that approximately two billion people currently suffer from infections with intestinal helminths in developing countries (7).

Despite the significant health burden that parasites cause, infections could be asymptomatic or show wide variations in clinical presentation, especially in protozoan parasites (8). Factors that affect disease severity remain poorly understood (8). Immune response contributes to protection from parasites; however, an increasing number of studies show that it is increasingly clear that the intestinal microbiota may have a significant influence on disease progression (8, 9).

Parasites usually enter the body through the oral fecal route and directly interact with the commensal bacteria of the intestine (8). Microbiota may increase resistance to parasitic infections at mucosal sites via changes in the composition of intestinal bacteria, and it may also alter systemic immunity to these parasites. Microbiota might also influence extraintestinal disease via many pathways, such as by the alteration of adaptive immunity and the development of T and B cell-mediated responses and bytheenhancement of innate immune pathways via trained immunity (10). Mechanisms underlying these extraintestinal effects are poorly understood. The focus of this review is the interactions between human microbiota and human parasites thatinfect the intestine and vagina or cause systemic infections.

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Intestinal Protozoan Parasites and Microbiota Interactions in Humans

Entamoeba histolytica, *Giardia intestinalis*, *Cryptosporidium* spp.,and *Blastocystis* spp. are the most common enteric protozoans that inhabit the intestinal mucosa and surround the intestinal microbiota. An increasing number of studies proposed that the clinical outcome of these parasitic infections could be shaped by host microbiota and host immune system (8).

The referenced human studies so far suggest that there is a strong interaction between the composition of the intestinal microbiota and mucosa-associated protozoan parasites. In a previous study, increased numbers of *Prevotella copri* were found in infants with *E. histolytica* infections (11). It is also observed that both *P. copri* and *Prevotella stercorea* were significantly decreased in asymptomatic infected adults (12). Another previous study suggested that elevated levels of *P. copri* might also be associated with severe inflammation and an increased risk of autoimmune disease and colitis (8).

In a similar study, it is reported that the patients who were protected from *Cryptosporidium* infection had an increased level of indole-producing bacteria, such as *Escherichia coli*, *Bacillus* spp., and *Clostridium* spp. In contrast, infected patients had an increased level of *Bacteroides fragilis*, *Bacteroides pyogenes*, *Prevotella bryantii*, and *Akkermansia muciniphila* (13).

A previous study of intestinal parasite infection in individuals from a rural area showed that there was a significant increase in the relative number of *Bifidobacterium* spp. in *G. intestinalis*-positive patients (14). Increased Clostridia levels but lower Enterobacteriaceae levels were observed in *Blastocystis*-positive subjects (15). All these studies suggested that the tested intestinal parasites may induce significant bacterial changes in the microbiota, and gut microbiota has also a potential influence on parasite infection outcome.

Trichomonas vaginalis, a mucosal protozoan parasite, which is the causative agent of trichomoniasis, is the most common non-viral sexually transmitted infection globally. It has been shown that variation in the clinical presentation of the disease is impacted by the composition of the vaginal microbiota consisting of low proportions of lactobacilli but a higher level of *Mycoplasma* spp., *Parvimonas* spp., and *Sneathia* spp. (16). It is also shown in another previous study that *Lactobacillus* species inhibit parasite interactions with human cells (17).

An emerging study suggests that protozoa may also alter host immunity to subsequent exposures (8). It is suggested that *Giardia* infection has been associated with protection from diarrhea. During this prospective study, it was observed that acute diarrhea occurred less often among *Giardia*-positive children than among children who were not infected with *G.intestinalis* (18).

A recent study in murine models provides a demonstration of how protozoan infection might provide protection from subsequent infections. *Tritrichomonas musculis* is a common murine commensal that has been shown to cause the expansion of adaptive T helper (Th) 1 cells and Th17 effector cells in the colonic mucosa. This expansion required the production of interleukin (IL)-18 by epithelial cells. *T. musculis* colonization showed significant protection from *Salmonella* infection-driven enteritis in an IL-18-dependent manner. However, colonization with *T. musculis* exacerbated the development of Tcell-driven colitis and resulted in the development of sporadic colorectal tumors in colonized mice (19). Combined, these studies revealed novel host–protozoan interactions that led to increased mucosal host defenses while also increasing the risk of inflammatory disease (8, 19).

***Plasmodium spp*. and Microbiota Interactions in Humans**

It is shown that not only intestinal parasites but also blood and tissue parasites can be affected by gut microbiota (8, 20). Researchers are investigating the effects of bacterial microbiota on the clinical variability of *Plasmodium* spp. infection. Approximately 60% of the population worldwide is at risk of infection with *Plasmodium* spp., which is the causative agent of malaria disease (21). Malaria kills approximately 660,000 people/year. However, the distribution of clinical malaria is also highly heterogeneous. Genetic differences, variation in exposure, and variance in immune response may not completely explain clinical variation (8, 21).

A recent study showed that the intestinal microbiota of a patient who became infected with *Plasmodium falciparum* had a significantly lower level of *Bifidobacterium* and *Streptococcus* species than that of subjects who did not become infected (22). This suggests that the alteration of the intestinal microbiota composition by probiotics may decrease the risk of *P. falciparum* infection in endemic areas.

Recently, the influence of the microbiota on *Plasmodium* infection was explored by Villorino et al., and significant differences in parasitemia level were observed between the genetically identical mice infected with *Plasmodium yoelii* from different vendors. Resistant mice exhibited higher numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. than susceptible mice. After cecal transplants to germ-free mice from resistant or susceptible mice, low and high parasite burdens were observed, respectively (23). Resistant mice exhibited higher antibody profile and higher CD4 T cells and B cells than susceptible mice. These findings suggest that the intestinal microbiota may shape the severity of malaria, and the composition of the gut microbiota may be an unidentified risk factor for severe malaria. The alteration of the gut microbiota might affect the host response to extraintestinal parasites (23).

It has also been shown that the gut microbiota has a systemic influence on serum metabolites in humans (8). These findings suggest that blood-stage parasites might also be influenced by serum metabolite changes induced by the microbiota. It is recently demonstrated that anti-α-gal antibodies confer protection against *Plasmodium* spp. infection in humans. Both *Plasmodium* spp. and *E. coli* O86:B7 (normally a member of an intestinal microbiome) express α-gal that produces protective anti-α-gal antibodies. Anti-α-gal antibodies, immediately after inoculation by *Anopheles* mosquitoes, target *Plasmodium* sporozoites for com-

plement-mediated cytotoxicity in the skin. Experiments showed that vaccination against α-gal antigen confers sterile protection against malaria in mice, suggesting that a similar approach may reduce malaria transmission in humans (24). This study is a very good example that gut microbiota has a systemic influence on serum metabolites in humans, and that probiotics-based malaria vaccines might be used in the near future.

Alteration of Gut Microbiota as a Therapy for Protozoan Infections

In the near future, microbiota studies will establish a more complete understanding of the variation in clinical presentations of parasitic protozoa infection and the effects of the microbiota on parasite survival and proliferation. These studies suggest that the alteration of individual components of the microbiota might provide cost-effective prophylactic treatment for parasite infection without using antiparasite agents (25). The alteration of the intestinal microbiota in model systems may also help to understand the role of immune factors in a clinical variation of parasitic disease.

Murine models provide a useful tool to explore host–microbiota–pathogen interactions (8). Currently, few in vitro and in vivo disease models provide us with a useful tool to understand interactions between infecting agents and components of the microbiota. For example, an invitro study demonstrated that the proliferation of *Giardia* trophozoites was significantly inhibited by *Lactobacillus johnsonii* La1 strain. The protective role of *L. johnsonii* La1 was confirmed by in vivo experiments with La1-treated gerbils (26). In another in vitro study, *Lactobacillus casei* and *Enterococcus faecium* were cocultured with *E.histolytica* and reduced parasite survival by 80%. An in vivo study demonstrated a link between decreased *Lactobacillus* spp. and amebiasis in humans (27).

It was previously described that lactobacilli may impact susceptibility to *T. vaginalis* infection (8). Even though the mechanisms underlying this effect are still unknown, protection might be explained by the inhibition of adhesion by the parasite to epithelial cells (17). In an in vitro study, *T.vaginalis* trophozoites and *Lactobacillus gasseri* ATCC 9857 were incubated in vaginal epithelial cells, and significant parasite adhesion inhibition was observed (17).

In another previous study, it is demonstrated that segmented filamentous bacteria (Gram-positive, spore-forming bacteria that were originally identified in the ilia of mice and rats) colonized mice are protected from experimental *E. histolytica* infection (28). It was also discovered that bone marrow-derived dendritic cells from segmented filamentous bacteria-colonized mice produced significantly higher levels of IL-23. This ILis responsible for induction of IL-17A and neutrophils, which are both important in immunity to the ameba (29). Transfer of bone marrow-derived dendritic cells from segmented filamentous bacteria-colonized mice provided protection from *E. histolytica* infection (28). This important study suggested that gut microbiota might alter the responsiveness of bone marrow-derived cells to the inflammatory response.

In an animal model of *Giardia* infection,theantibiotic alteration of the microbiota was shown to prevent CD8 T cell activation by *Giardia duodenalis* (20). One potential mechanism is that during infection, the parasite promotes the breakdown of the intestinal barrier. Translocation of luminal bacteria into the mucosa leads to the activation of CD8 T cells; therefore, reducing the bacterial load by antibiotic treatment may reduce this and prevent pathological CD8 T cell activation (20).

Intestinal Helminth Parasites and Microbiota Interactions in Humans

Helminth parasites usually live in adult form in the human intestine for a prolonged time. Helminths suppress host immunity to establish chronic infections and may impact on host responses against other pathogens (8). It is well known that both helminths and bacterial species had strong immunomodulatory effects. Our immune system has co-evolved together with large numbers of intestinal bacteria and helminths. Helminths were also described as themain selective force for selection of human genes associated with autoimmunity (30). Especially, chronic intestinal helminth infections have been documented to lower the severity of allergic and autoimmune disorders in humans (31). Interestingly, a study suggests that the eradication of helminths might be the reason for increasing number of chronic inflammatory diseases in Western countries.

Intestinal helminths can alter intestinal physiology, permeability, and mucous secretion that may impact to the microbiota. Currently, few studies have specifically addressed the impact of helminth infection on the microbiota. Conversely, the presence and composition of bacterial microbiota affect helminth colonization and persistence as well.

There are few reported microbiota studies from humans who were infected with helminths. In human populations, studies about the influence of helminth infection on microbiota composition and function have been only recently performed. A cross-sectional study was performed on 51 individuals who were infected by helminths from an endemic region (32). It is found that the helminth-colonized individuals had greater species richness in their stool sample than the uninfected individuals.

In a cohort study, *Schistosoma haematobium* infection-positive Zimbabwean children were found to have a significantly higher number of genus *Provetella* (33). In these subjects, microbiota composition did not revert even after helminth clearance with praziquantel treatment. This study suggested that childhood helminth exposure may have long-term effects on microbiota composition (33).

In another previous study, bacterial communities in stool samples of children living in a rural part of Ecuador were compared. A decreased in overall bacterial diversity was noted in helminth-infected children, especially in those coinfected with *Trichuris trichiura* and *Ascaris lumbricoides* (34). In an Australian study, stool samples from helminth-infected and non-infected individuals were compared (35). The authors reported a significant increase in bacterial diversity among individuals infected with any helminth species. In the same study, an increased number

of bacterial species belonging to the Paraprevotellaceae family was observed in subjects who were infected with *T. trichiura*. In another previous study, the impact of experimental infection with *Necator americanus* on intestinal bacterial communities in patients suffering from coeliac disease was observed (36). Stool samples from patients with coeliac disease both before and after infection with a low dose of *N. americanus* were compared, and increased bacterial diversity was noted in infected individuals (36). All these studies indicate that helminth infection may promote bacterial diversity.

Murine model experiments have clearly demonstrated that infection with helminth parasites may impact on the intestinal microbiota species composition. An increased number of Lactobacillaceae and Enterobacteriaceae species in the small intestine was observed during experimental chronic *Heligmosomoides polygyrus bakeri* infection in the duodenum of mice (37).

In a similar study, chronic infection with *Trichuris muris* (a mouse whipworm) leads to a reduced diversity of fecal bacterial species particularly within the *Bacteroides* spp., as well as an increase in the number of *Lactobacillus* spp. in the mice caecum (38).

In vitro animal studies showed that the presence and composition of bacterial microbiota may affect helminth colonization and persistence. A study showed that a murine nematode *H. polygyrus bakeri* (formerly named *Nematospiroides dubius*) caused mortality in germ-free mice but not in normal raised commercial mice (39). *L.casei* was shown to reduce adult worm burdens of *Trichinella spiralis* in murine models (40). Similarly, *Bifidobacterium* spp. strains provided protection against the helminth *Strongyloides venezuelensis* infection (9). These findings may suggest that intestinal bacteria prevent infection against parasitic helminths. However, it is still unclear whether direct interactions are occurring only between bacteria and helminths or indirectly via the host immunity. It is well known that the microbiota can modulate host immunity, and it may also impact on the host immune response against helminths. Germ-free mice exhibit smaller lymphoid tissues, decreased IgA levels but increasedIgE levels, and increased numbers of basophils and natural killer T cells (9, 41). Although the exact mechanisms still remain unclear, experimental studies needed to understand how microbiota and host immunity impact helminth infection.

A recent study has suggested that helminth infections have also an indirect effect on the modulation of mood and behavior in children via its effects on the alteration of the normal gut microbiota. Factors supporting this hypothesis are as follows: (1) gut microbiota playsa role on cognitive development (2), helminth infections can change gut microbiota composition and diversity, and (3) observed effect of helminth infection on cognitive development indicators (42).

Helminth infection can also have dramatic impacts on intestinal physiology, including increased fluid secretion, altered mucous production, and the infiltration of host immune cells that impact on bacterial communities via alterations to their habitat (9). Alterations in mucous cause dramatic consequences for bacterial growth and metabolism as many species use the mucous as an energy source (9).

Some studies indicated that helminth infection can also modify host metabolism (9). Bacterial-derived short-chain fatty acids (SCFAs) are well known to impact on host health by modulating immune function (43). Both helminth and protozoan parasites can also produce the SCFA acetate (44). Hamsters infected with the human hookworm *N.americanus* also showed extensively altered urinary metabolite levels that could be explained by changes in the intestinal microflora (45).

Helminths as a Therapeutic Agent for Infections?

Clinical studies and animal models have shown both human intestinal microbiota and helminths to be responsible for shaping human immunological responses. The alteration of the microbiota and using helminths to treat diseases could be a possible way to lower treatment cost (46). However, it is still unclear whether the immunemodulatory potential of helminths involves alterations of the microbiota via helminths providing the treatment.

Fecal transplantation from a healthy donor to diseased humans has been successfully used as a treatment for inflammatory bowel disease and *Clostridium difficile*-associated diarrhea (47). It is expected that helminth-induced alterations to intestinal bacterial communities may result in alterations to the severity of immune and metabolic diseases in humans. Helminth-based therapeutics, including infection with *Trichuris suis* or with *N. americanus*, has been used in allergy and inflammatory bowel disease (48). *N. americanus* was also used for treatment of coeliac disease, but no difference in symptoms was observed (49).

A couple of studies reported that helminth infection may provide protection against *Helicobacter*-induced gastric adenocarcinomas via preventing bacterial colonization of the stomach and reducing the number of neoplasticlesions (50). The experimental infection of *T. trichiura* in Macaques monkeys was reported to improve clinical symptoms in monkeys suffering from idiopathic chronic diarrhea (46). Mouse models and human studies have shown that chronic helminth infection regulates the host immune response and provides a beneficial action on allergic, autoimmune, and inflammatory disorders in humans (9).

CONCLUSION

The microbiota and parasites may interact with each other in different ways; microbiota may alternate parasite virulence, parasites may cause dysbiosis, and both parasites and microbiota may modulate host immunity. Recent studies have shown that the intestinal microbiotacan impact on clinical variation in parasitic infections. On the other hand, both human and animal studies indicate that both protozoa and helminth infections can impact on the intestinal microbiota. The exact mechanisms underlying the microbiota modulation of host immunity are not yet fully understood; however, it is becoming increasingly apparent that components of the microbiota may alter both innate and adaptive immune cell populations. The study of parasite interactions with the microbiota and the host immune system will help us to better understand the fundamental mechanisms of human

immunology. The exploration of interactions between the gut microbiota and parasites will provide additional information that will help with the diagnosis, treatment, and prevention of parasitic infections. It may also help with the treatment of inflammatory and autoimmune diseases.

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Original Research

Evaluation of Hypertension-Related Mortality in Turkey (2000–2014)

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ABSTRACT

Objective: Hypertension continues to be the leading risk factor for cardiovascular disease (CVD) and mortality worldwide. The purpose of the present study was to analyze the long-term trends of hypertension mortality in Turkey between 2000 and 2014 (for males and females).

Methods: Analyses were based on hypertension mortality data obtained from the Turkish Statistical Institute death database. Age-standardized mortality rates were calculated using direct standardization for each calendar year. We estimated the age-adjusted linear trend for annual percent change and average annual percent change (AAPC) with the corresponding 95% confidence interval (CI) using the joinpoint regression analysis. Furthermore, we conducted an age–period–cohort analysis to quantify recent time trends and to evaluate the significance of cohort and period effects.

Results: During the study period, a significant upward trend in the mortality of hypertension in Turkey is observed (AAPC=2.7%, 95% CI 1.9%–3.4%). The trend of hypertension mortality has increased in both males (AAPC=7.4%, 95% CI 3.0%–11.9%) and females (AAPC=8.7%, 95% CI 4.1%–13.5%). We found that the net drift rateswere 2.1% (95% CI 0.6%–3.6%) per year for males and 2.0% (95% CI 0.4%–3.7%) per year for females. According to longitudinal age curves, the mortality of hypertension increased with age in both males and females. The period and cohort effects are highly significant in both males and females.

Conclusion: Hypertension is one of the leading causes of mortality causing CVD. Knowing the risk factors and preventive methods could help to reduce hypertension-related mortalities.

Keywords: Age–period–cohort analysis, hypertension, join point regression analysis, mortality rate

INTRODUCTION

We live in a rapidly changing environment. Today, aging, rapid urbanization, and the globalization of unhealthy lifestyles are three important and powerful forces that shape human health globally. Hypertension is one of the major causes of disease burden worldwide. It is the leading CVD that causes at least 45% of deaths mostly owing to ischemic heart disease. In addition, it is responsible for 51% of deaths due to total stroke mortality (1). This cardiovascular major risk factor currently affects 1 billion people worldwide and causes heart attacks and stroke. Approximately 9 million people die annually due to hypertension (2).

Hypertension is more prevalent in low-income countries, but a large number of people are affected in high-income countries. As more people live in low-income countries and have weak health systems than other countries, the number of affected people is higher in those countries. People with undiagnosed, untreated, and uncontrolled hypertension are also higher in low-income countries than in high-income countries (3-5). The African region is the most prevalent region for hypertension (46% of adults, >25 years), whereas Americans have the lowest prevalence (35%) (6). Increased prevalence of hypertension, unhealthy diet, harmful use of alcohol, lack of physical activity, overweight, and permanent stress exposure are linked to population growth, aging, and behavioral risk factors. In 2008, approximately 40% of adults had hypertension in the 25 and over age group worldwide (6, 7).

Manyprevious studies have been conducted in Turkey on different perspectives of hypertension, define its prevalence and associated risk factors, but most of these studies are limited to specific areas (8, 9). There are limited data of large-scale surveys with nationally representative sample populations performed during 1991–2011 (10-13). All these studies were cross-sectional and did not evaluate the whole hypertension variation trends through a long period, and so the influence of changing demographics is unclear. Furthermore, there has been no wide analysis of the possible reasons underlying the temporal trends. To address these limitations, we aimed to investigate the long-term trends of hypertension mortality in Turkey between 2000 and

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2014, examining age-, period-, and cohort-specific effects by sex with the aid of the age–period–cohort (annual percent change, APC). Findings from our study could provide clues on thespecific etiologic inclusion on hypertension mortality in Turkey.

Demographers, sociologists, and epidemiologists attempt to distinguish age, period, and cohort effects. Owing to the disaggregation of this kind of data, it is likely to provide significant clues to the trends of different results, such as fertility, death, crime rates, and disease incidence. Age–period–cohort models are designed to predict the independent effects of age (A), period (P), and cohort (C) (14, 15).

The aim of an age–period–cohort analysis is intended to distinguish the contribution of age, period, and cohort effects on mortality. Although age, period, and cohort do not directly explain the variation in death rates, they are proxies for underlying biological, communal, and economical factors that affect mortality. Age effect reflects the biological and social processing of aging in a person and shows thedevelopmental changes throughout life. Period effect represents a complex set of historical cases and environmental effects, such as wars and economic crisis.Shifts in social, economic, healthier diet, or physical environments may in turn induce similar changes in the lives of individuals at a point in time. Cohort effect is the changes between groups of individuals experiencing a first event, such as birth or marriage in the same year (16).

METHODS

Analyses were based on hypertension mortality (ICD 10: I10–I15) data from the Turkish Statistical Institute death databasebetween 2000 and 2014 (17). The analysis was performed including central population of cities and villages between 2001 and 2008, but it was based on the total population number between 2009 and 2014.

Statistical Analysis

Age-standardized mortality rates per 100,000 people (using the World Health Organization (WHO) standard population)for each year were calculated using direct standardization. Changes in the age-standardized mortality rate were analyzed for hypertension (18). All analyses were performed separately for males and females as several aspects in hypertension differ between genders. First, these data were analyzed by joinpoint regression analysis(Joinpoint Trend Analysis, version 4.2.01; WA, USA) (19). This technique is generally used to module the time trends in mortality or incidence series in epidemiological studies. Joinpoint regression analysis was used to determine the significant change points in trends (i.e., "joinpoints").

The joinpoint regression model for observations, $(x_1, y_1),\ldots,(x_n,y_n)$, where $x_1\leq\ldots\leq x_n$without loss of generality, may be written as follows:

$E(y|x)=\beta_0+\beta_1 x+\delta_1(x-\tau_1)^+ +\ldots+\delta_k(x-\tau_k)+,$

where the τ_k 's are the unknown joinpoints. β_0 shows constant coefficient in equation, and β_1 shows slope coefficient (20).

The analysis begins with aminimum number of joinpoints and tests whether one or more joinpoints are statistically significant and should be added to the model. The number of joinpoints is decided by performing permutation tests with the correct level of asymptotic significance. This level of significance is found using Monte Carlo methods and Bonferroni correction (20).

In this research, each of detected trends is calculated by fitting a regression line to the logarithm of the rates, using year as an independent variable [ln(rate)=$\alpha+bx$], where x is year; APC was estimated as [100*(e^b–1)]. The parameters are allowed with a maximum of four joinpoints to enter the final model while having a minimum of 4 years between two joinpoints.The latest model shows the best fitting joinpoints where the rate changes significantly. Each joinpoint reports of a statistically significant change, an estimated APC, and AAPC that are computed along with its 95% confidence interval (95%CI). The AAPC is the geometric mean of the annual changes of all sections. In addition, the AAPC considers trend transitions (20).

Age–period–cohort analysis is based on a log-linear model for expected rates with additive effects for age, period, and cohort as follows:

$\rho_{p,\alpha}=\mu+\alpha_\alpha+\pi_p+Y_c,$

where α_a represents the effect of age, π_p the effect of period, and γ_c the effect of the *c*th birth cohort.

The general additional effects in the above equation can be divided into linear and nonlinear components.The two most useful are the age–period form (cross-sectional form):

$\rho_{p,\alpha}=\mu+\alpha_L-Y_L(\alpha-\bar{\alpha})+(\pi_L+Y_L)(p-\bar{p})+\acute{\alpha}_\alpha+\tilde{\pi}_{p+}\tilde{Y}_c,$

and the age–cohort form(longitudinal form):

$\rho_{c,\alpha}=\mu+\alpha_L-\pi_L(\alpha-\bar{\alpha})+(\pi_L+Y_L)(c-\bar{c})+\acute{\alpha}_\alpha+\tilde{\pi}_{p+}\tilde{Y}_c.$

Some estimable parameters and functions in the APC model:

μ, grand mean;

$\acute{\alpha}_\alpha$, $\tilde{\pi}_p$, $\tilde{Y}_c$, age, period, and cohort deviations;

α_L, π_L, longitudinal age trend;

α_L, Y_L, cross-sectional age trend;

π_L+Y_L, net drift;

$\mu+(\alpha_L-\pi_L)(\alpha-\bar{\alpha})+\acute{\alpha}_\alpha$, fitted longitudinal age-at-event curve;

$\mu+(\alpha_L-\pi_L)(\alpha-\bar{\alpha})+\acute{\alpha}_\alpha$, fitted cross-sectional age-at-event curve;

$\mu+(\pi_L-Y_L)(p-\bar{p})+\tilde{\pi}_p$, fitted temporal trends (21).

The referent age, period, and cohort are $\bar{\alpha}$=[(A+1)/2], $\bar{p}$=[(P+1)/2], and $\bar{c}=\bar{p}-\bar{\alpha}$+A, respectively, where P and A are the total numbers of the period and age groups.

Age–period–cohort analysis was performed for each gender to investigate age, period, and cohort effects onthe mortality rates of hypertension using a web tool, which was improved by the U.S. Department of Health and Human Services (Department of Biostatistics, Division of Cancer Epidemiology and Genetics, National Cancer Institutes, WA, USA) (22). The web tool fits the age–

Table 1. Time trends of age-standardized hypertension mortality rates in Turkey (2000-2014).

Joinpoints (years)	Time period	APC[1]	95%CI[2]	AAPC[3] 2000-2014	95%CI
Male					
1 joinpoint	2000-2005	−5.6	−15.3-5.3	7.4^	3.0-11.9
	2005-2014	15.3^	10.7-20.1		
Female					
1 joinpoint	2000-2005	−4.1	−14.4-7.6	8.7^	4.1-13.5
	2005-2014	16.6^		11.7-21.6	

[1]APC; [2]CI; [3]AAPC

^APC and AAPC are statistically significantly different from zero (two-sided p<0.05). CI: confidence interval: AAPC: average annual percent change: APC: annual percent change

period-cohort model and calculates parameters and predictable functions. The parameters are a combination of the observed hypertension rate and the functions that define the relationship between age, period, and cohort. The web tool also calculates a series of statistical hypothesis tests (Wald test) that determine whether the mortality due to hypertension is statistically significantly different from age, period, and cohort factors.

Net drift (annual percentage change of the expected age-adjusted rates over time) indicates the overall log-linear trend by period and cohort; the longitudinal age curve (expected age-specific rates in reference cohort$c_0$adjusted for period effects) in the mortality rates, which is the fitted longitudinal age-specific rates in reference cohort; cross-sectional age curve displays the fitted longitudinal age-specific rates in reference period (expected age-specific rates in reference period$p_0$adjusted for cohort effects); and local drifts (annual percentage change of the expected age-specific rates over time) can be generated from log-linear regressions. The web tool can also calculate the rate ratios. The period RR demonstrates the period relative risk adjusted for age and nonlinear cohort effects in a period versus reference period. Similarly, the cohort RR indicates the cohort relative risk adjusted for age and nonlinear period effects in a cohort versus reference period.In all APC analyses, the reference groups were the central age group, central period, and central cohort group (22). Ages included in the model were categorized into seven 5-year age groups (45–49, 50–54, 55–59, 60–64, 65–69, 70–74, and ≥75 years) since hypertension-related mortality had few cases under the age of 45 years. Years of mortality were divided into three 5-year calendar periods (2000–2004, 2005–2009, and 2010–2014). Finally, nine birth cohorts were obtained (1925, 1930, 1935, 1940, 1945, 1950, 1955, 1960, and 195). For all analysis in the present study, a bilateral p<0.05 was considered statistically significant.

RESULTS

A total of 109,615 people were involved in the research. Of the 109,615 individuals, 38% (n=41,751) were males, and 62% (n=67,864) were females. While the mortality rate of hypertension in females was 55% in 2000, it was increased to 63% in 2014. On the other hand, this ratio was reverse for males that was decreased from 45% to 37% in the same years. The average standardized or age-adjusted hypertension-related mortality rate in Turkey from 2000 to 2014 was found to be 9.20/100,000 people (8.60 for males and 9.80 for females). The hypertension-related mortality rate was female predominant, with the female-to-male rate ratio in age-standardized rate ranging from 0.89 to 1.23 across calendar years. The result of the joinpoint regression analysis, the APC for each trend, and the AAPC in both genders is shown in Table 1. For both genders, the hypertension-related mortality rates decreased during the period 2000–2005 (not statistically significant); however, there was a statistically significantdramatical increase throughout the period 2005–2014. With regard to gender, during the period 2000–2014, the AAPCs of the hypertension-related mortality rates were 7.4% in males and 8.7% in females (steadily increased).

During the study, local drift values showing APCs at mortality rates by gender for specific age groups are displayed in Figure 1. We saw local drift effects in similar patterns in both genders. The local drifts are highly significant in both males and females (male: χ^2:188.257, degrees of freedom (df): 6 and female: χ^2:227,289;df:6); local drift values increase by approximately 0%/year between males aged 55–59 years, approximately 6%/yearamong males aged 70–74 years, and approximately 18%/year among males aged ≥75 years; the local drift values increase from approximately 0%/year among females aged 60–64 years, approximately 6%/year among females aged 70–74 years, and approximately 18%/year among females aged ≥75 years.

Longitudinal age curves of hypertension-related mortality rates by gender are illustrated in Figure 2. As illustrated, the mortality of hypertension increased with age in both males and females. The risk of hypertension mortality increased monotonically in males until 62 yearsand then yielded significantly elevated increases for ≥65 years. The longitudinal age curves in females show similar overall patterns as males.

The estimated period and cohort effects by gender are shown in Figures 3 and 4, respectively. The period (male: χ^2:88.18, df:2 andfemale: χ^2:57.96, df:2) and cohort effects (male: χ^2:427.13, df:8 and female: χ^2:665.01, df:8) are highly significant in both males and females. We observed period effects in similar patterns for both genders, which shifted downwards since the period 2000–2007 and then turned upwards since the early 2008s with a significantly increased risk.

Figure 1. Local drift values for hypertension mortality rates. Age group-specific annual percent change (%) in the mortality rates of hypertension and corresponding 95% confidence intervals by gender

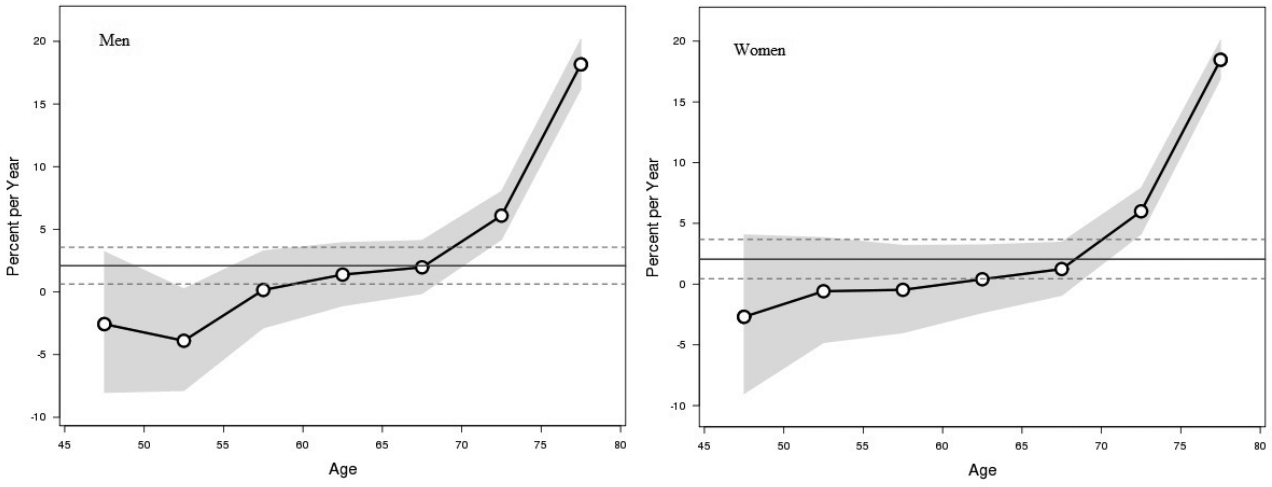
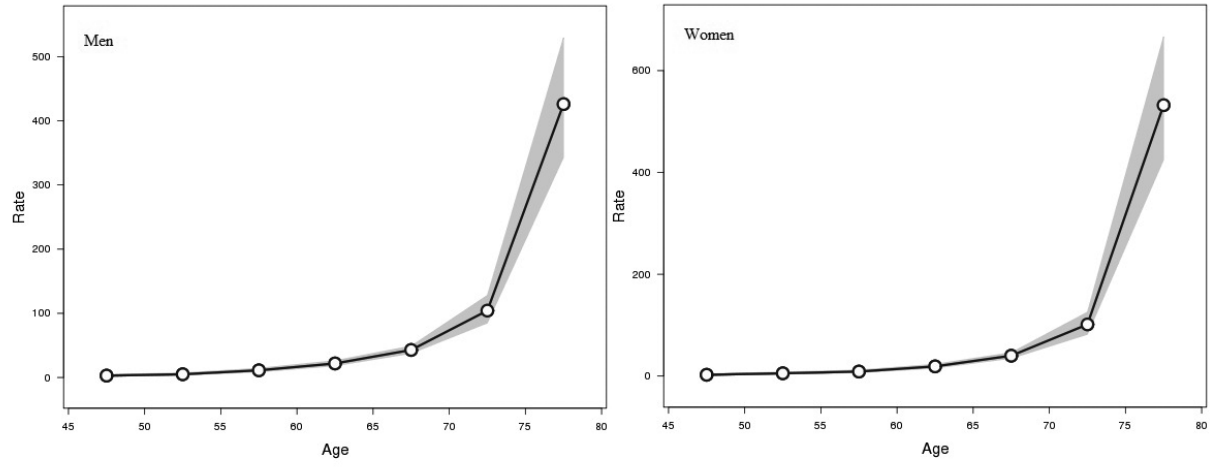


Figure 2. Longitudinal age curves of hypertension mortality rates. Longitudinal age curve of the mortality rates (1/100,000) of hypertension and corresponding 95% confidence intervals by gender



In general, the risk of hypertension increased with cohort in both genders. The cohort effects remained stable for the first several birth cohorts, followed by upwards inflation for males and females born after themid-1940s. Relative risk increased from 1925 to 1945, but then decreased.

DISCUSSION

The worldwide upward trend in mortality due to hypertension has been documented in the last decade. The present study included 109,615 cases of hypertension-related mortality recorded in the Turkish Statistical Institute death database between 2000 and 2014. To the best of our knowledge, this is the first study to investigate the trends of hypertension-related mortality in Turkey and to analyze age, period, and cohort effects by gender using the age–period–cohort analysis.

Our results indicated that there was a dramatic increase in age-standardized hypertension-related mortality rate (6.33–16.05) in Turkey. Using joinpoint regression analysis, a steady significant increase in both genders between 2000 and 2014, especially between 2005 and 2014, is observed. This significant increase may be related with the technology that made our lifestyles worse. More sedentary lifestyles, increase in the number of obese people, unhealthynutrition (e.g., fast food, frozen, salty, and fried foods), and increase in tobacco use would be the main factors that caused the increase in hypertension-related deaths. This increase alarmed the Ministry of Health, and in 2015, with a circular, sugar and salt were eliminated from the tables of cafes, restaurants, and dining halls, and so on (23).

Regarding the research by Kung and Xu (24), in the USA, the hypertension-related mortality rate for females is higher than

Figure 3. Period effects on hypertension mortality rates. Period effects obtained from age–period–cohort analysis for mortality rates of hypertension and corresponding 95% confidence intervals by gender

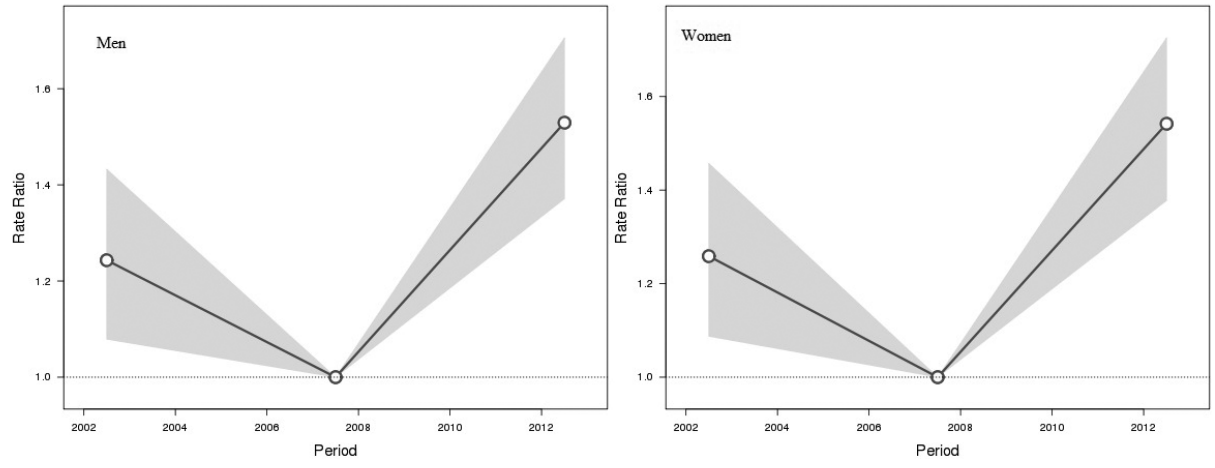
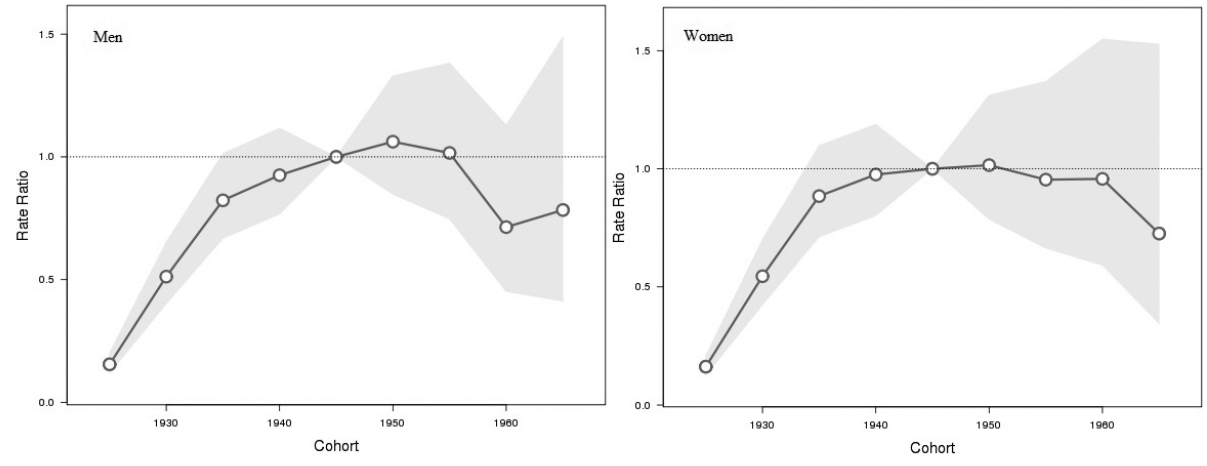


Figure 4. Cohort effects on hypertension mortality rates. Cohort effects obtained from age–period–cohort analysis for mortality rates of hypertension and corresponding 95% confidence intervals by gender



that for males aged ≥85 years, but more than that for females <84 years. Throughout the period 2000–2013, the rates for both females and males continued to increase until 2013, but progressed much more slowly (24). Similarly, the mortality rate was also higher in older females than in males in our study, especially after 2008. Through this period (especially in 2008), the mortality rates of hypertensive females were higher in the 75 and over age group, whereas there was a decrease in the other age groups. This similarity could probably be the result of higher prevalence of obesity and increasing CVD prevalence in females. In addition, the increase in sedentary lifestyle as females become oldercan be another risk factor. On the other hand, males are more active than femalesin Turkey, which makes the biggest difference in prevalence between the two gendersin older ages (6).

Aging is the unchanging risk factor for both genders. The results from age–period–cohort analysis showed that the curves of age and period effects increased, but generally decreased cohort effects.

Age is the most important risk factor for hypertension among a series of demographic factors. The risk of death from hypertension varies from different life stages.The present study and other studies (24, 25) showed that the rate of hypertension-related mortality increased with age. As the aging population increases, chronic diseases, especially hypertension, become more important and the most probable reason for mortality worldwide.Populations globally are rapidly aging, andthe prevalence of hypertension increases with age (25, 26). This condition reflected to cohort effects. In our study, the cohort

effects (relative risk) increased upwards for both genders from 1925 to 1945 (especially those born after the mid-1940s). This indicated that currently, people born in this period are aged between 73 and 93 years, which is overthe expected life duration. Regarding WHO,72.0 years was the average life expectancy at birth of the global population in 2016 (27). In these ages, not only hypertension but also other coexisting diseases would be the reasons of this upward increase.

The study data of Trabzon on hypertension stated that hypertension is very common, and it is an important health problem in adult population in Trabzon (8). Hypertension rate is nearly 30% (similar with the previous study), and the difference between male and female and urban and rural disappears.There was an 11% decrease in the prevalence of age-standard hypertension from TURDEP-I to TURDEP-II. The smoking rate in Turkey increased to42% in 12 years (10). We can explain this by decreasing smoking rate and by strong legislative regulations on salt restriction. However, the decrease in hypertension prevalence did not affect the mortality rates; on the contrary, the mortality rate of hypertension increased.

Currently, media, especially the internet, is the main source of much knowledge including information about health. This makes many people aware of the risks of their hypertension, and they take care about preventive methods, for example, restricting salt, exercising, and paying attention to body mass index.

In the present study, time trend analysis on hypertension mortality rates was ecological descriptive analysis at population levels without deduction at individual levels.

The PatenT2 study showed that the prevalence of hypertension in Turkish adults was stable between 2003 and 2012, but showed a significant improvement in treatment and control rates in hypertension awareness (25). This would reflect a decrease in hypertension mortality rates in the future.

As an important public health problem, hypertension is still underlining the need for a specific national program to improve the detection and control of hypertension in this country. This national initiative should develop well-organized programs, guidelines, and policies to facilitate hypertension prevention, detection, awareness, and treatment.

These hypertension mortality rates showed us that hypertension is still an epidemic problem in our country. We are still not aware of this common problem and still cannot treat satisfactorily.As we know, there are many behavioral risk factors for development of hypertension includingfood consumption with too much salt and fat, not eating enough fruit and vegetables, and harmful alcohol levels; physical inactivity and lack of exercise; and poor stress management. In addition, there are a number of metabolic factors that increase the risk of heart disease, stroke, renal failure, and other hypertension complications, such as diabetes, high cholesterol, and excessively overweight or obese. Regarding these risk factors, we can argue the changes in hypertension mortality and morbidity rates.

Cohort effects reflectenvironmental factors, such as war, famine, inflation, and recession, that can affect the subject in their first years of life. The period effect refers to the impacts of environmental, nutritional, and sociological changes on the later years of an individual's life. These changes include lifestyle alternations, such as addiction, smoking, and diet changes during adolescence and adulthood. Among both male and female participants of the PatenT2 study, hypertension was associated with an increased frequency of well-known risk factors, as we mentioned above, such as self-reported parental hypertension, smoking, diabetes, obesity, and a sedentary lifestyle. During the past 10 years, the mean body mass index increased by 1.1 kg/m^2 in females and by 1.57 kg/m^2 in males in Turkey. The prevalence of obesity also increased from 20.5% to 28.7%. High salt consumption is also a major health problem in Turkey. Bread is an important component of meals in this country (28, 29).

Systolic blood pressure and salt intake were significantly correlated in normal weight subjects ($r=0.257$, $p<0.01$). The Turkish population consumes a large amount of salt, affecting blood pressure; there was a positive correlation between salt intake and blood pressure. In recent years, the Turkish Ministry of Health eliminatedsalt from the tables of restaurants. For this reason, efforts to restrict sodium are very important in the management of hypertension as part of national and global health policies (26, 28). Even a small decrease in sodium intake in the daily diet caused asignificant decline in blood pressure levels.

Our study has some limitations. One of the limitations of the study was the use of official published mortality data. Regarding this data, we could obtain village and urban data separatelyuntil 2008. However, after this year, as only the total numbers were provided, we could analyze the mortality rates for the whole population after that year. In addition, not obtaining the information about the comorbid diseases of the patients and other hypertension risk factors, such as nutrition, body mass index, medications, dyslipidemia, and smoking status, are the limitations of the present study.

CONCLUSION

Hypertension is one of the leading causes of mortality. Although hypertension-related mortality rates change, we know that age is the main risk factor for all periods. Knowing the risk factors and preventive methods could help to reduce hypertension-related mortalities. Screening for hypertension, especially in high-risk groups, such as elderly women, could help to reduce CVD. This will allow for early intervention and follow-up on populations atrisk, thus, contributing to improve strategies for reducing hypertension burden.

Ethics Committee Approval: The article is not requires ethics committee approval. The data used in this article are taken from the official website of the Turkey Statistical Institute.

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Original Research

Surgical Treatment Results in Unilateral Wilms Tumor: Experience from a High-Volume Pediatric Oncology Center in Turkey

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ABSTRACT

Objective: The aim of the present study was to evaluate the clinical characteristics, factors affecting treatment approach, and long-term outcome of patients with Wilms tumor.

Methods: We identified the demographic features, mode of presentation, applied treatments, and long-term outcomes of 88 patients treated between 1990 and 2011 at Hacettepe University İhsan Doğramacı Children's Hospital according to the Turkish Pediatric Oncology Group protocol. Data were analyzed using SPSS program, and chi-square test was used for statistical analysis.

Results: The study included 88 patients (50 females and 38 males) with a mean age at presentation of 3±2.48 years. Patients were classified as stage 1 (n=35, 39.8%), stage 2 (n=16, 18.2%), stage 3 (n=17, 19.3%), and stage 4 (n=20, 22.7%). Pathological examination of tumors revealed favorable histology in 76 (86.4%) patients and unfavorable histology in 10 (11.4%) patients. Forty-nine (55.6%) patients received preoperative chemotherapy, and patient's age at diagnosis and physical examination findings influenced the decision of the administration of preoperative chemotherapy ($p<0.05$). Of the 88 patients, 25% aged <1 year and 75% aged between 3 and 5 years received preoperative chemotherapy. The palpated mass was crossing the midline in 20.5% of patients who were subjected to primary surgery. Tumor ruptured in 5.6% of patients intraoperatively. Long-term prognosis of patients was as follows: 68 (83.9%) children were cured and 13 (16%) children died due to recurrences and metastases. Survival rates reached 100% in stage 1 and 2 patients but decreased to 75% and 50% in stage 3 and 4 patients, respectively.

Conclusion: Age at presentation and physical examination findings are significant in surgical planning. Stage is the most important prognostic factor. Patients with Wilms tumor are treated with low complication and high survival rates due to multidisciplinary treatment approach at our institution.

Keywords: Child, preoperative chemotherapy, survival, Wilms tumor

INTRODUCTION

Wilms tumor is the most common renal tumor of childhood (1). Prognosis is excellent among patients with localized disease and favorable histology by virtue of multicenter collaborative studies (2). Safer reduction of chemotherapeutic agents and radiotherapy doses with improved surgical technique leads to fewer short- and long-term complications and longer life expectancy in these patients. However, metastatic, recurrent disease, unfavorable histology, and patients with syndromes or genetic predispositions to Wilms tumor still stand as a therapeutic challenge (3). The aim of the present study was to identify the characteristics of patients treated with unilateral Wilms tumor and the factors affecting treatment approach and prognosis from a surgical standpoint.

METHODS

The study was approved by the Hacettepe University Senate Ethics committee at 02.07.2012 with the issue number 429. All patients who presented with a diagnosis of unilateral Wilms tumor between 1990 and 2011 were identified. Patients whose surgical treatments and chemotherapy and radiotherapy applications were performed entirely in Hacettepe University İhsan Doğramacı Children's Hospital were included in the study. Exclusion criteria comprised patients receiving a part of medical or surgical treatment in another medical center. Eighty-eight patients with unilateral Wilms tumor were eligible for the study. The treatment algorithm of each patient was discussed by the multidisciplinary pediatric oncology team and planned according to the Turkish

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Pediatric Oncology Group (TPOG) protocol (4). Parental consent was not necessary since this was a retrospective chart review.

Age, gender, associated syndromes, presenting signs and symptoms, details of surgical intervention, complications of surgery, pathology results, stage, histology, metastasis, chemotherapy and radiotherapy regimens, and prognosis of these patients were reviewed retrospectively. Patient-related factors affecting the treatment algorithm (upfront surgery vs. chemotherapy) and survival were identified.

Statistical Analysis

Statistical analysis was performed by Statistical Package for the Social Sciences version 16.0 (SPSS Inc.; Chicago, IL, USA). Variables were analyzed by chi-square test. A p value <0.05 was accepted as statistically significant.

RESULTS

The study included 88 patients consisting of 50 girls and 38 boys with a mean age of 3±2.48 years. The most common presenting symptoms were abdominal distention as noted by the caregivers (n=53, 60.2%), abdominal pain (n=17, 19.3%), blood in urine (n=12, 13.6%), fever (n=8, 9.1%), and vomiting (n=5, 5.7%). The mass extending over the midline was palpated in 35.2% (n=31) of children. Hypertension was detected in 9 (10.2%) patients. The incidence of inguinoscrotal pathology was 6.8%. Among the Wilms tumor predisposing syndromes, two patients were diagnosed with Beckwith-Wiedemann syndrome (BWS), one with WAGR and another with Silver Russel syndrome. Tumor thrombus in the renal vein or inferior vena cava was present in six patients at the time of diagnosis. Stage distribution according to the TPOG and aforementioned data is represented in Table 1.

Of the 88 children, 49 (55.7%) were administered preoperative chemotherapy and 39 (44.3%) underwent upfront nephrectomy. Partial nephrectomy was performed in one patient with BWS and solitary kidney. Cavatomy and thrombectomy were performed in four patients with tumor thrombus. Lymph nodes were sampled from the paracaval and paraaortic region in 28 (31.8%) and from the renal hilum in 25 (28.4%) patients. Tumor ruptured during surgery in five (5.6%) patients, and two of them were operated without preoperative chemotherapy. Pathology results revealed favorable histology in 86.4% and unfavorable histology in 11.4% of cases.

Table 1. Clinical characteristics of patients with Wilms tumor

Characteristics	Patients	n (%)
Gender	Male	38 (43.2)
	Female	50 (56.8)
Presenting symptom	Abdominal distention	53 (60.2)
	Abdominal pain	17 (19.3)
	Blood in urine	12 (13.6)
	Fever	8 (9.1)
	Vomiting	5 (5.7)
Physical examination findings	Mass limited to one side of the abdomen	57 (64.8)
	Mass extending over the midline	31 (35.2)
	Hypertension	9 (10.2)
	Inguinoscrotal pathologies	6 (6.8)
Stage distribution	Stage 1	35 (39.8)
	Stage 2	16 (18.2)
	Stage 3	17 (19.3)
	Stage 4	20 (22.7)
Presence of tumor thrombus in the inferior vena cava or renal vein		6 (6.8)
Associated syndromes	BWS	2 (2.3)
	WAGR	1 (1.1)
	Silver Russel syndrome	1 (1.1)

BWS: Beckwith–Wiedemann syndrome; WAGR: Wilms tumor, aniridia, growth retardation

Table 2. Distribution of upfront surgery versus chemotherapy with respect to age

	<1 year n (%)	1–2 years n (%)	3–5 years n (%)	≥6 years n (%)	Total n (%)
Upfront chemotherapy	2 (25)	16 (45.7)	24 (75)	7 (53.8)	49 (55.7)
Upfront surgery	6 (75)	19 (54.3)	8 (25)	6 (46.2)	39 (44.3)
Total	8 (100)	35 (100)	32 (100)	13 (100)	88 (100)

p=0.022

Table 3. Distribution of upfront surgery versus chemotherapy with respect to physical examination findings

	Mass extending over the midline n (%)	Mass limited to one side of the abdomen n (%)	Total n (%)
Upfront chemotherapy	23 (46.9)	26 (53.1)	49 (100)
Upfront surgery	8 (20.5)	31 (79.5)	39 (100)
Total	31 (35.2)	57 (64.8)	88 (100)

p=0.01

Table 4. Prognosis according to stage of patients

Stage	Cure n (%)	Exitus n (%)	Total n (%)
1	33 (100)	0 (0)	33 (100)
2	14 (100)	0 (0)	14 (100)
3	12 (75)	4 (25)	16 (100)
4	9 (50)	9 (50)	18 (100)
Total	68 (83.9)	13 (16)	81 (100)

Seven patients were lost to follow–up
p=0.01

The administration of preoperative chemotherapy was more common among older patients than among infants aged <12 months (Table 2). Upfront surgery was preferred over chemotherapy more commonly in patients when the palpated mass is limited to one side of the abdomen (Table 3).

Local recurrence in the tumor bed was seen in 10 patients. Pathological examination revealed unfavorable histology in one of them. Metastatic involvement of the lymph nodes was present in 2 of 3 children who had lymph node sampling. Among the possible causes of recurrence, capsule invasion was noted in four, and tumor rupture during surgery was seen in two patients. Recurrent tumor was resected in four children.

Among the 81 patients with long-term follow-up, 83.9% survived the disease, and 16% died due to the disease and complications of treatment. Seven patients were lost to follow-up. Survival was not affected by gender or administration of preoperative chemotherapy (p=0.587 and p=0.086, respectively). Survival rates were not different across age groups (p=0.562), but all infants aged <1 year survived the disease. All patients with syndromes predisposing to Wilms tumor had complete remission of disease. Stage was the only statistically significant parameter affecting prognosis (p=0.01). All of the children with disease stages 1 and 2 were cured. Cure rates decreased to 75% and 50% in patients with stage 3 and 4 diseases, respectively (Table 4).

DISCUSSION

Wilms tumor is the most common renal malignancy of childhood. Currently, survival reaches 90% in localized disease and 70% in metastatic cases. This success is attributed to the work of multicenter collaborative studies conducted by international consortiums (2). This effort leads to a reduction in chemotherapy and radiotherapy regimens and standardization of surgical treatment. In the present study, we reviewed our 20-year experience in treating patients with Wilms tumor from a surgical perspective.

Wilms tumor can be seen at any age, but it is most common in the third year of life. In our study, the mean age of the patients was similar to other studies (5, 6). The major complaints are nonspecific symptoms, such as abdominal pain, distention, vomiting, and hematuria (7). Hypertension and genitourinary anomalies can be observed during physical examination (8). We observed that the frequency of presenting symptoms was not different from the ones stated above in our patients.

There are two large clinical groups conducting trials for Wilms tumor: Children's Oncology Group (formerly NWTSG), which advices upfront surgery, and Société Internationale d'Oncologie Pédiatrique (SIOP), which supports upfront chemotherapy. Tumors are staged before chemotherapy in the former and after chemotherapy in the latter group. Upfront surgery carries the risk of tumor rupture, relapse, and advancement of stage. On the other hand, chemotherapy before tissue diagnosis carries the risk of unnecessary treatment for benign tumors, inadequate regimen for renal tumors other than nephroblastoma, downstaging, and therefore inadequate chemotherapy afterwards (2, 7, 9). TPOG established a national protocol, and patients are evaluated individually for upfront surgery or chemotherapy by the local multidisciplinary pediatric oncology team (4). Upfront surgery and chemotherapy approaches were almost equally distributed among our patients. Given the fact that our institution is a referral center for pediatric oncology patients in Turkey, we encounter more patients with advanced tumor stage, associated syndromes, and surgically challenging tumors. Patients with

these features are directed to upfront chemotherapy to prevent surgical complications. Attitude toward upfront surgery among infants aged <12 months can be justified by the fact that congenital mesoblastic nephroma is frequent in this age group (10). Physical examination finding of a mass at the time of diagnosis was an important determinant of upfront surgery or chemotherapy decision in our study. Chemotherapy decreases tumor size and risk of rupture (11). Tumor rupture rate was 5.6% among all patients in our study. This rate increases to 15.3% in NWTSG and decreases to 2.2% in SIOP (12, 13). From the perspective of surgical complications, our approach is reasonable.

Documentation of surgical details has utmost importance. Tumor rupture; spill; extension to adjacent organs; palpation of tumor thrombus in the renal vein or vena cava, perihilar, paraaortic, and paracaval lymph node sampling; and exploration of contralateral kidney and solid organs for metastasis if performed should be written in detail (1). These facts can change the stage and treatment algorithm of the patient toward a more or less aggressive way. Ehrlich et al. (12) found that many deviations from the guidelines are observed during surgery including failure to sample lymph nodes and tumor spill in the NWTS-5 surgical quality assessment. In our series, lymph node sampling was performed in 31.8% of our patients. This rate is much lower than NWTSG results and accepted as a self-criticism. Recurrent tumor in patients with stage 2 tumor was higher than expected among children without lymph node sampling in NWTS-5 (14). Fortunately, none of our patients with stage 2 disease had recurrent tumor.

Anaplasia, stage, lymph node status, and chromosomal abnormalities are the most important prognostic parameters in children with Wilms tumor (7). Age and gender did not appear to affect survival, but many studies including ours found an increased survival trend in infants diagnosed before the age of 1 year. Small abdominal cavity and apparent mass result in early diagnosis and localized disease. Anaplasia is also rare in this age group (1, 10). Cure rates were not different in patients with upfront surgery or chemotherapy. Although overall prognosis is excellent, it is much lower in stage 3 and 4 diseases. In our study, stage was the only significant parameter on survival. The small number of patients appears to be the reason of statistical insignificance in other parameters. Our finding is supported with other studies (15). We argue that survival is not affected by the mode of treatment but particular characteristics of the patient and disease itself.

CONCLUSION

Preference over upfront surgery or chemotherapy should be done in a case-based manner. Although this approach does not have an effect on prognosis, it can reduce surgical complications in patients with Wilms tumor. Further prospective studies are necessary to compare results.

Ethics Committee Approval: Ethics committee approval was received for this study from the Hacettepe University Senate ethics committee at 02.07.2012 with the issue number 429.

Informed Consent: Due to the retrospective design of the study, informed consent was not taken.

Peer-review: Externally peer-reviewed.

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Original Research

Effects of Gabapentin on Carrageenan-Induced Inflammation, Acute Phase Reactants and Gastric Mucus Secretion in Rats

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ABSTRACT

Objective: Gabapentin (GBP), which was first developed as an anticonvulsant agent, has recently gained great attention considering its pain relieving and anti-inflammatory effects. The aim of the present study was to investigate the anti-inflammatory effect of GBP on carrageenan (CAR)-induced paw edema and its gastric side effects by determining gastric mucus secretion in rats.

Methods: Paw edema was induced with 1% CAR immediately before intraperitoneal saline, GBP (10 or 30 mg/kg), and diclofenac (DIC) (5 mg/kg) injections. Paw thickness was measured hourly during 6 h. Serum cytokine levels were determined using enzyme-linked immunosorbent assay. For evaluation of gastric mucus secretion, saline, GBP, and DIC were administered orally for 10 days. On day 10, stomachs were removed, and gastric mucus of the glandular part was evaluated spectrophotometrically.

Results: Both doses of GBP reduced paw thickness at 6 h ($p<0.05$). GBP decreased interleukin (IL)-1β and tumor necrosis factor (TNF)-α levels similarly to DIC, whereas it increased IL-10 levels less than DIC ($p<0.05$). Both doses of GBP and DIC decreased gastric mucus secretion compared with control ($p<0.05$).

Conclusion: Our results suggest that GBP produces anti-inflammatory effect comparable with DIC. However, the effects of GBP on gastric mucus secretion were not better than those of DIC.

Keywords: Anti-inflammatory effect, carrageenan-induced inflammation, cytokine levels gabapentin, gastric mucus

INTRODUCTION

Gabapentin (GBP), a congener of gamma-aminobutyric acid, was first produced as an anticonvulsant agent. GBP has recently gained great attention considering its pain relieving and anti-inflammatory effects (1-3). In addition to its usage in epilepsy, it is also indicated for management of postherpetic neuralgia (4) and is found to be effective in clinical studies of inflammatory hyperalgesia or diabetic neuropathy and trigeminal neuralgia (1-3). Furthermore, the experimental antinociceptive effect of GBP on neuropathic or chronic pain was reported (5-8). GBP was proposed to present antinociceptive activity in rat formalin test (7) that involves the stimulation of inflammatory and sensitization processes (9, 10) and also to have analgesic effects in other inflammatory pain tests (11, 12).

Inflammation, a defensive mechanism of the body in response to tissue damage, includes complicated biochemical and cellular mechanisms evoked by inflammatory molecules, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which stimulates the chemotaxis of neutrophils (12). The influence of proinflammatory cytokines in nociception is also reported (13, 14).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used in the treatment of various painful and inflammatory conditions. However, gastric adverse effects, such as ulcer formation, limit clinical use (15, 16). NSAIDs produce gastrointestinal damage via several mechanisms, such as reducing the secretion of cytoprotective components and causing injury on mucosal epithelium (15, 16). Gastric mucus secretion is one of the major mechanisms of mucosal protection (17).

Considering the antinociceptive effects of GBP and the association of pain and inflammation, it may be suggested that GBP may produce an anti-inflammatory effect. Therefore, we aimed to evaluate the anti-inflammatory activity of GBP in carrageenan (CAR)-induced paw edema in rats by measuring paw thickness and release of proinflammatory cytokines IL-1β and TNF-α and

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anti-inflammatory cytokine IL-10 and then to explore the gastric side effect of GBP on gastric mucus secretion and compare all these effects with an NSAID diclofenac (DIC).

METHODS

Animals

All experimental procedures were approved by the Eskişehir Osmangazi University local ethics committee for animal experimentation (no.: 502, date: 11.02.2016). Male Wistar rats (250–350 g, 14–16 weeks) were kept in standard conditions at 23°C±1°C and 12:12 h light: dark cycle.

Drugs

Drugs were dissolved in saline (0.9% NaCl). CAR (Alfa Aesar, Karlsruhe, Germany) was administered to rats by intradermal (i.d.) route at a dose of 100 µl. GBP (Sigma-Aldrich, St. Louis, MO, USA) 10 or 30 mg/kg was administered intraperitoneally (i.p.) for evaluation of anti-inflammatory effect and via gastric lavage for gastric mucus evaluation. An NSAID, DIC sodium (Sigma-Aldrich, St. Louis, MO, USA) 5 mg/kg was administered i.p. and via gastric lavage for gastric mucus evaluation.

Experimental Design

Two sets of experiments were performed as follows:

In the first experiment, the anti-inflammatory effect of GBP was assessed by CAR-induced paw edema. The experimental groups were designed as follows (n=7 per group):

1. Control group: 100 µl saline (i.d.)+saline (i.p.),
2. CAR group: 100 µl CAR (i.d.)+saline (i.p.),
3. GBP 10 mg/kg group: 100 µl CAR (i.d.)+10 mg/kg GBP (i.p.),
4. GBP 30 mg/kg group: 100 µl CAR (i.d.)+30 mg/kg GBP (i.p.),
5. DIC 5 mg/kg group: 100 µl CAR (i.d.)+5 mg/kg DIC (i.p.).

In the second experiment, the effect of GBP on gastric mucus secretion was evaluated. Drugs were administered via gastric lavage during 10 days.
The experimental groups were designed as follows (n=7 per group):

1. Control group (saline),
2. GBP 10 mg/kg group,
3. GBP 30 mg/kg group,
4. DIC 5 mg/kg group.

Rats in groups used in the first and second experiments were different.

CAR-induced Paw Edema

Carrageenan (100 µl) was injected by i.d. route per rat into the right hind paw. The rats were injected with i.p. 10 or 30 mg/kg GBP immediately after CAR injection. Saline was given to the control group. A 5 mg/kg DIC was used as a reference anti-inflammatory agent. Paw thickness was measured using a vernier caliper just before (0 h) and after the injection of CAR at 1, 2, 3, 4, 5, and 6 h (18, 19).

Cytokine Assay

Intracardiac blood samples at 6 h were kept at −80°C until analysis. IL-1β, TNF-α, and IL-10 levels in serum were determined using eBioscience Enzyme-Linked ImmunoSorbent Assay (ELISA) kits (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction.

Determination of Gastric Mucus Secretion

Saline, GBP 10 or 30 mg/kg, or DIC 5 mg/kg was administered via oral gavage for 10 days. The animals were not given food, but water was accessible overnight before the animals were sacrificed on day 10. Their stomachs were removed immediately, and then their gastric lumens were rinsed with saline. The amount of the gastric mucus of the glandular part was evaluated spectrophotometrically at 605 nm by Alcian blue dye binding method (20). Briefly, the stomachs were removed, rinsed with saline, weighed, and incubated for 2 h in 20 mL of 0.1% of w/v Alcian blue in 0.16 mol/l sucrose solution buffered with 0.05 mol/l sodium acetate (pH 5.8). The gastric tissue was immersed in 0.5 mol/l $MgCl_2$ solution for 2 h to extract the mucus-bound dye. The blue extract was shaken with diethyl ether, and the resultant emulsion was centrifuged at 5000 g for 10 min. The supernatant was measured at 600 nm by spectrophotometry. Data are presented as µg Alcian blue/g tissue.

Statistical Analysis

Data were analyzed using statistical Package for the Social Sciences (SPSS) statistical package software version 21.0 (SPSS IBM Corp.; Armonk, NY, USA). Results of paw edema and gastric mucus concentration were expressed as mean±standard error (SEM). Data of paw edema were analyzed using one-way repeated measures ANOVA, and gastric mucus secretion was analyzed using one-way ANOVA. Data of cytokine levels were analyzed using independent Kruskal–Wallis test. One-way ANOVA or Kruskal–Wallis tests were used based on the normal or non-normal distribution of data, respectively. Dunnett's C or Tukey's tests were used as post hoc tests. Cytokine levels were expressed as median and percentiles (25%–75%). A p-value <0.05 was considered as statistically significant.

RESULTS

The Effects of GBP on CAR-induced Paw Thickness

Paw thickness did not differ between the groups at all time points, except the CAR group (Figure 1). CAR significantly increased paw thickness at 4, 5, and 6 h compared with other groups ($p<0.05$). Both doses of GBP significantly decreased paw thickness, and this effect was comparable with DIC (Figure 1). Results were expressed as mean±SEM.

The Effects of GBP on Serum Cytokine Levels

Carrageenan significantly increased IL-1β and TNF-α levels compared with control ($p<0.05$). GBP decreased IL-1β and TNF-α levels compared with the CAR group ($p<0.05$). These effects of GBP were similar to DIC (Figures 2, 3). GBP increased IL-10 levels compared with the CAR group and control ($p<0.05$). DIC increased IL-10 levels more than the GBP groups ($p<0.05$) (Figure 4). Results were expressed as median and percentiles (25%–75%).

Figure 1. The effects of GBP on CAR-induced paw edema. *p<0.05 compared with the control, GBP 10 mg/kg, GBP 30 mg/kg, and DIC 5 mg/kg groups. Results were expressed as mean±SEM
CAR: carrageenan; GBP 10: gabapentin 10 mg/kg; GBP 30: gabapentin 30 mg/kg; DIC 5: diclofenac sodium 5 mg/kg

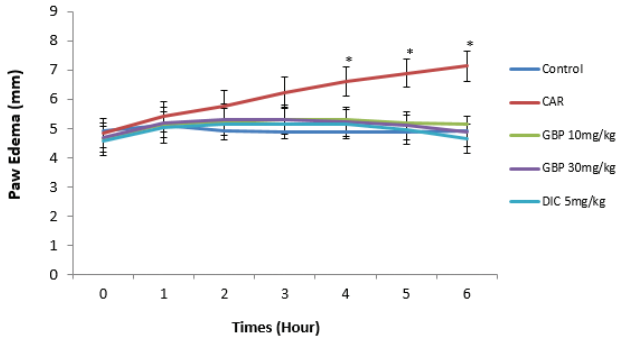


Figure 2. The effects of GBP on serum IL-β levels. *p<0.05 compared with the control group, +p<0.05 compared with the CAR group. Results were expressed as median and percentiles (25%–75%)
CAR: carrageenan; GBP 10: gabapentin 10 mg/kg; GBP 30: gabapentin 30 mg/kg; DIC 5: diclofenac sodium 5 mg/kg

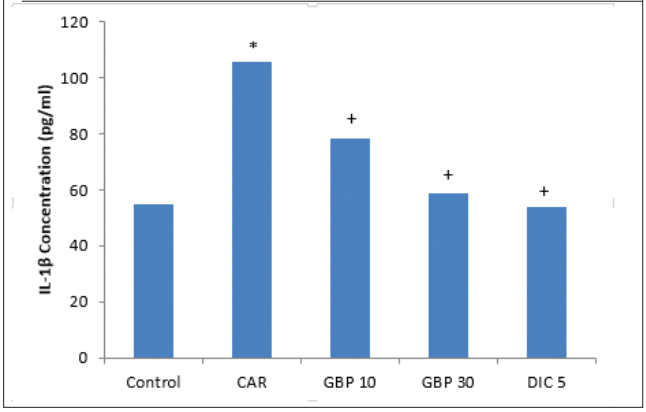


Figure 3. The effects of GBP on serum TNF-α levels. *p<0.05 compared with the control group, +p<0.05 compared with the CAR group. Results were expressed as median and percentiles (25%–75%)
CAR: carrageenan; GBP 10: gabapentin 10 mg/kg; GBP 30: gabapentin 30 mg/kg; DIC 5: diclofenac sodium 5 mg/kg

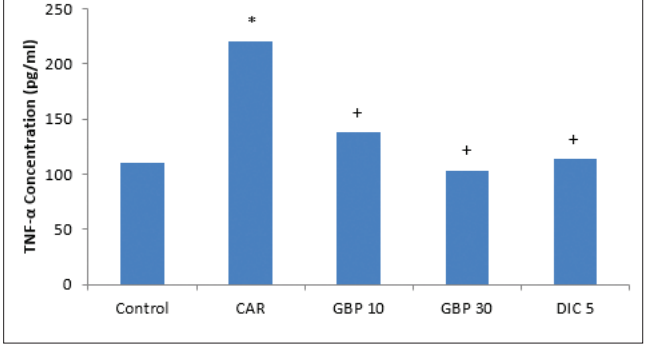


Figure 4. The effects of GBP on serum IL-10 levels. *p<0.05 compared with the control group, +p<0.05 compared with the CAR group, #p<0.05 compared with GBP 10 mg/kg and GBP 30 mg/kg groups. Results were expressed as median and percentiles (25%–75%)
CAR: carrageenan; GBP 10: gabapentin 10 mg/kg; GBP 30: gabapentin 30 mg/kg; DIC 5: diclofenac sodium 5 mg/kg

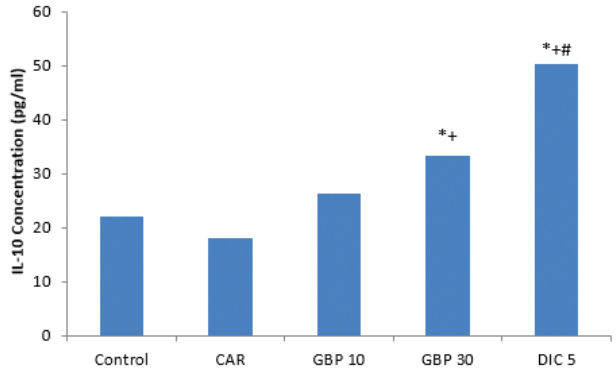
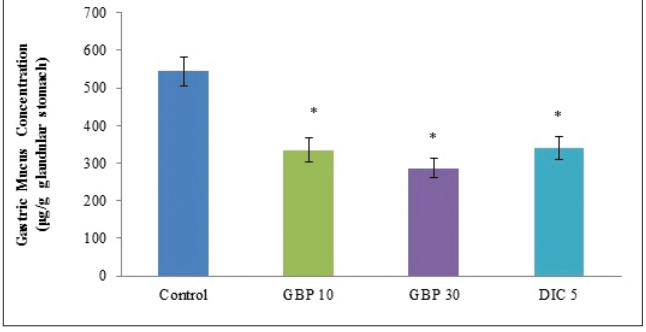


Figure 5. The effects of GBP on gastric mucus secretion. *p<0.05 compared with the control group. Results were expressed as mean±SEM
Control: saline; GBP 10: gabapentin 10 mg/kg; GBP 30: gabapentin 30 mg/kg; DIC 5: diclofenac sodium 5 mg/kg



The effects of GBP on Gastric Mucus Secretion

Both doses of GBP decreased gastric mucus secretion compared with control (p<0.05). DIC also reduced gastric mucus secretion compared with control (p<0.05) (Figure 5). However, GBP and DIC did not differ according to the effects on gastric mucus secretion (Figure 5). Results were expressed as mean±SEM.

DISCUSSION

Gabapentin, an antiepileptic agent, has been indicated for relief of neuropathic pain. It was reported that GBP exhibited central and peripheral antinociceptive effects via nitrergic and serotonergic pathways (21). Considering its antinociceptive effects, the present study was designed to investigate if GBP induced gastropathy that is seen with the use of analgesic NSAIDs. DIC, which is an NSAID, was used as a reference drug to compare its effects on inflammation and gastric mucus secretion with GBP.

The present study demonstrated that GBP reduced inflammation induced by CAR injection in rats supported with the reduced IL-1β and TNF-α with GBP 10 and 30 mg/kg doses. Moreover, there was a significant enhancement in the levels of anti-inflammatory cytokine IL-10 with only GBP 30 mg/kg compared with the CAR and control groups.

Therefore, our results point out that GBP is an anti-inflammatory drug against inflammatory painful stimulus. Our results agree with previous studies showing that GBP exhibited anti-inflammatory activity on CAR-induced paw edema (12, 22). We also found that the anti-inflammatory effect of GBP was independent from dose and comparable with DIC. These results were comparable with previous studies showing that low doses of GBP, 1 mg/kg i.p. administration of GBP in mice and 25 and 50 mg/kg subcutaneous administration of GBP in rats, reduced the inflammatory response in CAR-induced paw edema that was also less than those of one of the NSAIDs indomethacin (12, 22). Generally, data suggest that GBP is not better than NSAIDs for reducing inflammation.

Carrageenan-induced paw edema is a widespread model to study the anti-inflammatory effects of drugs. Local injection of CAR causes the release of various proinflammatory and inflammatory (i.e., prostaglandins (PGs), leukotrienes, histamine, bradykinin, and TNF-α) cytokines (23). Recently, this model is used for localized inflammatory pain.

The inflammatory response to CAR consists of three phases. The first phase is intervened by histamine and 5-hydroxytryptamine, and then second, the kinin-mediated phase occurs. The third phase is associated with PGs, especially those of the E series. Cyclooxygenase (COX) enzymes catalyze the production of PGs and thromboxanes from arachidonic acid. NSAIDs inhibit COX enzymes that are associated with their clinical benefit in the management of pain and inflammation (4, 24). Thus, CAR-induced paw edema has been a fundamental method in the development of NSAIDs and new COX inhibitors (23).

Both doses of GBP decreased gastric mucus secretion compared with control. DIC also reduced gastric mucus secretion compared with control. However, there was no significant difference between GBP 10 and 30 mg/kg and DIC 5 mg/kg with regard to the effects on gastric mucus secretion.

In addition, in the literature, GBP was found to prevent gastric mucosal lesions induced by an NSAID drug indomethacin, and it was suggested that GBP showed a gastroprotective effect (22). This finding partly disagrees with the results of our study. We observed that GBP reduced gastric mucus secretion, indicating a probability of gastric mucosal damage formation. However, this effect was comparable with NSAID DIC. On the other hand, we did not investigate the effect of GBP on gastric mucosal lesions as gastric ulcer index. NSAIDs are commonly used in the treatment of inflammatory diseases, rheumatoid arthritis, osteoarthritis, dysmenorrhea for relief of pain, inflammation, and fever. Long-term use of NSAIDs is accompanied with severe gastrointestinal adverse effects, such as mucosal erosions, bleeding, ulcer formation, perforations, and intestinal obstructions (9).

There are three main mechanisms of NSAID-induced gastrointestinal complications: inhibition of COX-1 enzyme and gastroprotective PGs, increase of membrane permeabilization, and proinflammatory mediators (10). COX enzymes consist of two isoforms with different functional properties (12). COX-1 is constitutively resident and contributes to the maintenance of physiological functions of gastric mucosa by catalyzing PG synthesis that protects gastric tissue against gastric acid and produces HCO_3 and sustains gastric mucosal blood flow (13, 14). NSAID-induced gastropathy mainly occurs with the inhibition of COX-1. COX-2, an inducible isoform, is triggered by cell damage, proinflammatory cytokines, and tumoral factors (20).

In the present study, we observed that both doses of GBP reduced gastric mucus secretion. On the other hand, this reduction was similar to the gastric mucus reduction as seen with DIC.

CONCLUSION

We observed that the anti-inflammatory effect of GBP was supported by decreased serum levels of IL-1β and TNF-α. In addition, especially GBP 30 mg/kg increased anti-inflammatory cytokine IL-10 levels in serum. Therefore, our results exerted that GBP is an anti-inflammatory drug against inflammation. GBP may provide at least some benefit, but it might not be recommended over NSAIDs. We suggest that GBP reduces gastric mucus secretion; however, this was not worse than DIC.

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Original Research

Evaluation of Microsatellite Instability in Colorectal Adenomas and Carcinomas by Immunohistochemistry and a Comparison of Histopathological Features

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ABSTRACT

Objective: Approximately 15% of sporadic colorectal carcinomas (CRCs) develop along the microsatellite instability (MSI) pathway. In this study, we compared the MLH1, MSH2, Ki-67, and p53 immunostaining properties with histopathological features of colorectal adenomas and CRCs.

Methods: A total of 102 cases were selected, including 50 adenomatous polyps, 25 adenocarcinomas, 10 adenocarcinomas with mucinous component, 14 mucinous adenocarcinomas, and three signet-ring cell carcinomas. The tissues were stained for MLH1, MSH2, p53, and Ki-67 primary antibodies.

Results: Negative staining was observed for MLH1 in 25% and MSH2 in 3.8% of all CRC cases. Compared with adenocarcinoma not otherwise specified (NOS), mucinous adenocarcinomas showed weaker staining for MLH1, which was statistically significant. There was also a statistically significant difference between adenocarcinoma NOS and signet-ring cell carcinomas in terms of negative staining for MLH1. A total of 69.2% of the MLH1-negative cases were high-grade. There was a statistically significant relationship between the histological grade and MLH1 negativity. A positive correlation was found between the grade of dysplasia and p53 staining intensity and Ki-67 proliferation index. No negative staining was observed for MLH1 and MSH2 in any of the adenomatous polyps.

Conclusion: For the histopathological examination of CRCs, in the presence of mucinous and poorly differentiated morphology, tumor-infiltrating lymphocytes and Crohn-like inflammatory response, immunohistochemical staining for MLH1, and MSH2 antibodies may be useful in the detection of tumors showing MSI.

Keywords: Adenomatous polyps, colorectal carcinomas, immunohistochemistry, microsatellite instability

INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of morbidity and mortality worldwide. CRC, representing approximately 9.7% of newly diagnosed carcinomas worldwide (1), is the fourth most common type of carcinoma in males after lung, prostate, and stomach carcinomas. Furthermore, in females, it is the third most common type of carcinoma after breast and cervical carcinomas in the world (1). Colorectal adenomas are relatively common lesions. The incidence increases after 50 years age. Colorectal adenomas are recognized as precursors in the majority of CRC cases (1-4).

Molecular events involving both genetic and epigenetic abnormalities occur during the development of CRCs. At least two genetic pathways have been identified. These are the chromosomal instability pathway and the microsatellite instability (MSI) pathway (1). Approximately 15% of sporadic CRCs and almost all of the CRCs in patients with hereditary nonpolyposis colorectal carcinoma develop with the MSI pathway. Indirect immunohistochemical MSI diagnosis can be given as a result of developing specific antibodies against mismatch repair proteins, which are gene products of the mismatch repair system (1). A high level of agreement was found between the immunohistochemically detected MLH1 and MSH2 expressions and the MSI status determined by molecular methods (5-8).

In this study, the MLH1, MSH2, Ki-67, and p53 staining properties of colorectal adenomas and CRCs were evaluated and compared with the histopathological features.

METHODS

Endoscopic polypectomy materials and colorectal resections (right hemicolectomy, left hemicolectomy, transverse colectomy, sigmoidectomy, and low-anterior resection) that were sent

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to the Department of Pathology, Recep Tayyip Erdogan School of Medicine between 2011 and 2016 were obtained from our pathology reports. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the Ethics Committee approval. Patients who were diagnosed with CRCs and adenomatous polyps were enrolled in the study after obtaining their written informed consent. Among these cases, a total of 102 cases were selected, including 20 tubular adenomas, 20 tubulovillous adenomas, 10 villous adenomas, 25 adenocarcinomas not otherwise specified (NOS), 10 adenocarcinoma with mucinous component, 14 mucinous adenocarcinomas, and three signet-ring cell carcinomas selected from our laboratory archive. Patients receiving neoadjuvant therapy and those who had a familial cancer history were not included in this study. The H&E stained slides of the cases were reviewed again, and the most suitable paraffin blocks were identified. Sections taken at a 4-micrometer thickness from selected paraffin blocks were placed onto poly-L-lysine coated slides. Primary antibodies to MLH1 (mouse monoclonal, clone G168-15, isotype IgG1/kappa, ready for use, Biocare Medical, USA), MSH2 (mouse monoclonal, clone FE11, isotype IgG1/kappa, Biocare Medical, USA), Ki-67 (Rabbit monoclonal, clone SP6, isotope rabbit IgG, Thermo Scientific, Fremont, CA, USA), and p53 (mouse monoclonal, clone BP53-12, IgG2a/kappa, ScyTek Laboratories, Logan, USA) were used for immunohistochemical staining with automated an IHC/ISH staining instrument (Roche, Ventana, Benchmark, TX , USA). Normal human tonsil tissue (for MLH1, MSH2, and Ki-67) and colon adenocarcinoma (for p53) were used as external positive controls. The results were evaluated independently by two pathologists with an Olympus BX51 light microscope.

Statistical Analysis

The Statistical Package for Social Sciences version 13.0 (SPSS Inc.; Chicago, IL, USA) statistical software was used to analyze the data. The one-way analysis of variance, Student's t test, and Mann-Whitney U test were used to compare the descriptive statistical measures (mean, standard deviation), as well as the quantitative data. The normal distribution of the data was assessed by the one-sample Kolmogorov-Smirnov test. A chi-squared test and Fisher's exact test were used for comparison of the qualitative data. Correlation coefficients and statistical significance were calculated by the Kendall Correlation test for the inter-variable interrelationships. P-values <0.05 were considered as significant.

Morphological Evaluation

Current World Health Organization criteria were used for adenoma and carcinoma diagnosing and typing, and for tumor differentiation and tumor grading (1). According to these criteria, the tumors were grouped as well differentiated, moderately differentiated, poorly differentiated, and undifferentiated. The following criteria for grading based on the gland formation alone were used: low-grade, greater than or equal to 50% gland formation; high-grade, less than 50% gland formation. These grading systems were not used for signet-ring cell carcinoma and mucinous carcinomas, as these tumors were accepted as high-grade (1). The TNM staging system by the American Joint Committee on Cancer and the International Union Against Cancer were used for pathologic tumor staging. The intratumoral lymphocytic response (tumor-infiltrating lymphocytes) was assessed as 1 (none), 0-2 (mild to moderate 0-2 per high-power [X400] field), 3 (marked three or more per high-power field). Peritumoral lymphocytic response (Crohn-like response) was rated as 1 (none), 2 (mild to moderate), and 3 (marked). A stromal reaction in the tumor was classified as mild desmoplasia [1], moderate desmoplasia [2], and severe desmoplasia [3]. Histopathologic parameters such as tumor necrosis, perineural invasion, lymphovascular invasion, and tumor nodule presence were classified as present or absent.

The caecum, ascending colon, hepatic flexure, and transverse colon were determined as the right colon; the splenic flexure, descending colon, sigmoid colon, and rectum were determined as the left colon.

Immunhistochemical Analysis

The nuclear staining for Ki-67, p53, MLH1, and MSH2 was considered positive. The nuclear staining for MLH1 and MSH2 was considered positive, whereas the absence of staining was considered as negative staining. Tumors showing the loss of nuclear MLH1 or MSH2 expressions were classified as MLH1- or MSH2-negative. Nuclear staining in normal epithelial cells, lymphocytes, and stromal cells was used as a positive internal control for MLH1 and MSH2. In addition, the tonsillar tissue and normal bowel tissue were used as an external positive control on the slide. A single scoring system was used for both Ki-67 and p53. According to this scoring, a 0%-10% staining was accepted as G0, 11%-50% staining as G1, 51%-75% staining as G2, and 75% staining as G3.

RESULTS

Of the 50 polypectomy cases, 21 were female (42%), and 29 (58%) were male. The mean age was 61.94 years. The age of the oldest patient was 84 and the youngest 33 years. 86% of the patients were over 50 years. Twenty polyps were tubular adenomas (40%), 20 tubulovillous adenomas (40%), and 10 villous adenomas (20%). Twenty-eight of the polyps (56%) had low-grade dysplasia, and 22 (44%) had high-grade dysplasia. The age and gender distribution, dysplasia grade, diameters, MSH2, MLH1, Ki-67, and p53 staining characteristics of polyps are summarized in Table 1. A positive correlation was found between dysplasia and p53 staining ($p<0.001$, $r=0.516$). A positive correlation was found between dysplasia and the Ki-67 proliferation index ($p<0.001$, $r=0.458$). It was determined that 100% of the tubular adenomas contained low-grade dysplasia, 70% of tubulovillous adenomas had high-grade dysplasia, and 80% of villous adenomas had high-grade dysplasia. There was a statistically significant relationship between the histologic type of adenomas and dysplasia grade ($p<0.001$). All adenomas were positively stained with MLH1 and MSH2 (Figures 1-4).The mean age in CRC cases was 62.4 years, with the maximum age being 86 years and the youngest age being 36 years. Twelve of the patients were under 50 years old. 42.3% of CRC cases were female, and 57.7% were male. The age distribution and localization of CRC cases and p53, Ki-67, MLH1, and MSH2 staining results are summarized in Table 2. The comparison of the MLH1 and MSH2 staining of CRCs with histopathological findings (differentiation, tumor grade, necrosis, lymphatic invasion, perineural invasion, stromal reac-

Table 1. Relationship between age, gender, and histopathological findings in adenomatous polyps n (%)

	Tubuler adenoma	Tubulovillous adenoma	Villous adenoma	p
Number/ Percentage	20 (40%)	20 (40%)	10 (20%)	
Gender				0.691
Female	9 (45%)	9 (45%)	3 (30%)	
Male	11 (55%)	11(55%)	7 (70%)	
Age				
≥50 years	19 (95%)	15 (75%)	9 (90%)	
<50 years	1 (5%)	5 (5%)	1 (10%)	
Configuration				<0.001
Sessile	20 (100%)	9 (45%)	8 (80%)	
Pedunculated	11 (55%)	2 (20%)		
Mean size	0.47 cm	1.53 cm	1.78	0.001
Displasia				<0.001
Low grade	20 (100%)	6 (30%)	2 (20%)	
High grade		14 (70%)	8 (80%)	
p53				
G1	13 (65%)	–	–	
G2	7 (35%)	16 (80%)	9 (90%)	
G3	–	4 (20%)	1 (10%)	
Ki-67				
G1	11 (55%)	1 (5%)	–	
G2	9 (45%)	14 (70%)	8 (80%)	
G3	–	5 (25%)	1 (20%)	
MLH1 staining	Positive staining	Positive staining	Positive staining	
MSH2 staining	Positive staining	Positive staining	Positive staining	

MLH1: mutL homolog 1; MSH2: mutS homolog 2

Figure 1. MLH1 positively stained tubular adenoma (x400)

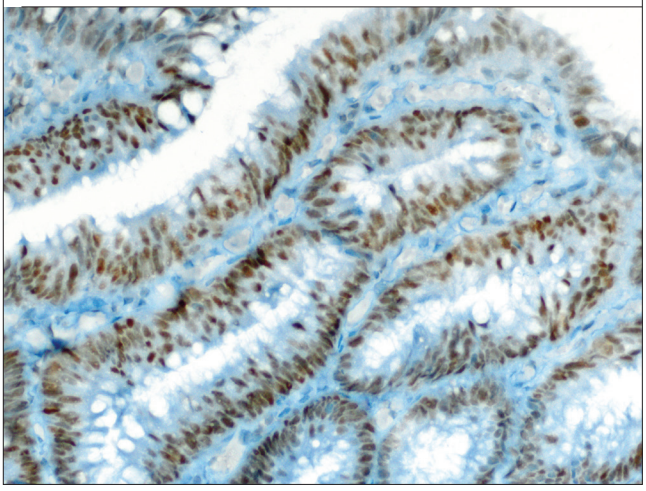


Figure 2. MSH2 positively stained tubular adenoma (x400)

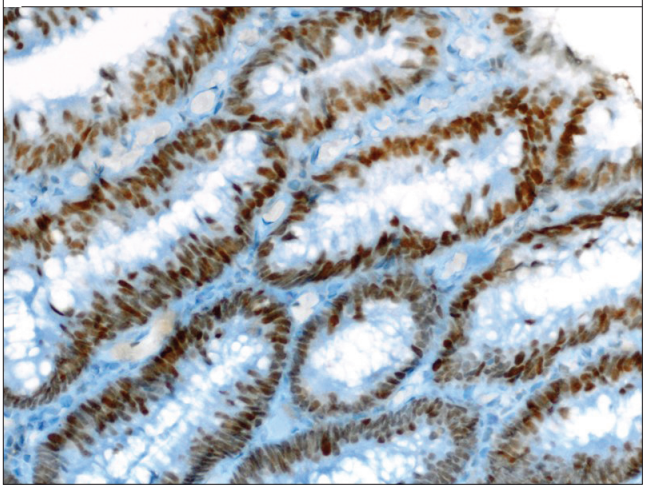
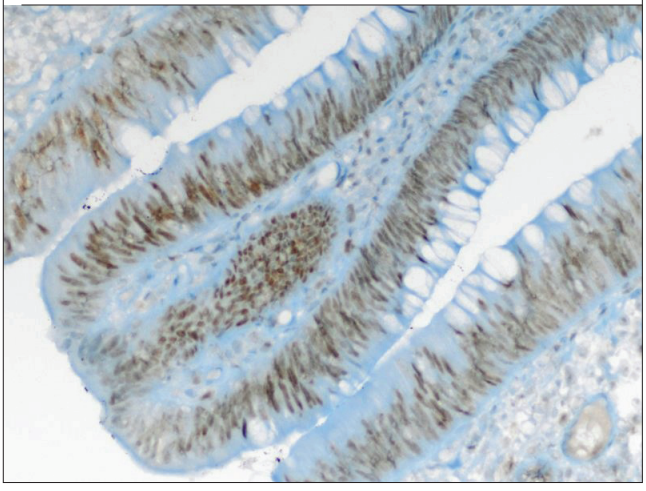


Figure 3. MLH1 positively stained villous adenoma (x400)



tion, intratumoral lymphocytic response, Crohn-like response, and pathologic stage) is summarized in Table 3. No statistically significant difference was observed between adenocarcinoma, NOS (negative staining in 4%), and adenocarcinoma with mucinous component (negative staining in 30%) in terms of negative staining with MLH1 (p=0.061). There was no statistically significant difference between adenocarcinoma NOS (no negative staining) and adenocarcinoma with mucinous component (negative staining in 20%) in terms of negative staining with MSH2 (p=0.076) (Figures 5-9). There was a statistically significant difference between adenocarcinoma NOS (negative staining in 4%) and mucinous adenocarcinomas (negative staining in 50%) in terms of negative staining with MLH1 (p=0.001) (Figure 10). Positive staining with MSH2 was detected in all adenocarcino-

Table 2. Relationship between cancer types and other histopathological findings n (%)

	Adenocarcinoma, NOS	Adenocarcinoma with mucinous component	Mucinous adenocarcinoma	Signet-ring cell carcinoma
Age				
≥50 years	16 (64%)	9 (90%)	12 (85.7%)	3 (100%)
<50 years	9 (36%)	1 (10%)	2 (14.3%)	–
Localization				
Cecum	2 (8%)	3 (30%)	5 (35.7%)	1 (33.3%)
Ascending colon	4 (16%)	2 (20%)	1 (7.1%)	–
Hepatic flexure	2 (8%)	–	–	1 (33.3%)
Transverse colon	1 (4%)	–	2 (14.3%)	–
Splenic flexure	3 (12%)	–	1 (7.1%)	–
Descending colon	1 (4%)	1 (10%)	3 (21.4%)	–
Sigmoid colon	10 (40%)	2 (20%)	2 (14.3%)	–
Rectum	2 (8%)		2 (20%)–	1 (33.3%)
MLH1				
Positive staining	24 (96%)	7 (70%)	7 (50%)	1 (33.3%)
Negative staining	1 (4%)	3 (30%)	7 (50%)	2 (66.7%)
MSH2				
Positive staining	25 (100%)	8 (80%)	14 (100%)	3 (100%)
Negative staining	–	2 (20%)	–	–
p53				
G0	5 (20%)	–	2 (14.3%)	–
G1	4 (16%)	2 (20%)	6 (42.9%)	–
G2	2 (8%)	2 (20%)	1 (7.1%)	1 (33.3%)
G3	14 (56%)	6 (60%)	5 (35.7%)	2 (66.7%)
Ki-67				
G1	–	–	2 (14.3%)	–
G2	8 (32%)	3 (30%)	7 (50%)	1 (33.3%)
G3	17 (68%)	7 (70%)	5 (35.7%)	2 (66.7%)

Adenocarcinoma NOS: adecarcinoma not otherwise specified; MLH1: mutL homolog 1; MSH2: mutS homolog 2

mas NOS, mucinous adenocarcinomas, and signet-ring cell carcinomas. There was a statistically significant difference between adenocarcinoma NOS (negative staining in 4%) and signet-ring cell carcinomas (66.7% negative staining) in terms of negative staining with MLH1 (p=0.023). There was no statistically significant difference between adenocarcinomas with mucinous component (30% negative staining) and mucinous adenocarcinomas (50% negative staining) in terms of the MLH1-negative staining (p=0.421). Adenocarcinomas with mucinous component (negative staining in 20%) and mucinous adenocarcinomas (negative staining were not observed) showed no statistically significant difference between each other in terms of the MSH2-negative staining (p=0.163) (Figure 11). There was no statistically significant difference in terms of the MLH1-negative staining between mucinous adenocarcinomas (negative staining in 50%) and signet-ring cell carcinomas (negative staining in 66.7%) (p>0.05) (Figure 12). There was no statistically significant difference between adenocarcinomas with a mucinous component (30% negative staining) and signet-ring cell carcinoma (66.7% negative staining) in terms of the MLH1-negative staining (p=0.510). There

Table 3. Comparison of the MLH1 and MSH2 staining with other histopathological findings in CRCs n (%)

	MLH1+	MLH1-	P	MSH2+	MSH2-	P
Gender						
Female	15 (38.5%)	7 (53.8%)	0.331	22 (44%)	-	0.502
Male	24 (61.5%)	6 (46.2%)		28 (56%)	2 (100%)	
Differentiation						
Well differentiated	3 (7.7%)	-		3 (6%)	-	
Moderately differentiated	25 (64.1%)	4 (30.8%)		27 (54%)	2 (100%)	
Poorly differentiated	3 (7.7%)	-		3 (6%)	-	
Not applied	8 (20.5%)	9 (69.2%)		17 (34%)	-	
Histological grade						
Low	28 (71.8%)	4 (30.8%)	0.021	30 (60%)	2 (100%)	0.517
High	11 (28.2%)	9 (69.2%)		20 (40%)	-	
pT						
pTis	1 (2.6%)	-		1 (2%)	-	
pT1	-	-		-	-	
pT2	6 (15.4%)	2 (15.4%)		8 (16%)	-	
pT3	28 (71.8%)	8 (61.5%)		34 (68%)	2 (100%)	
pT4a	4 (10.3%)	2 (15.4%)		6 (12%)	-	
pT4b	-	1 (7.7%)		1 (2%)	-	
Necrosis						
Present	26 (66.7%)	8 (61.5%)	0.747	33 (66%)	1 (50%)	1.000
Absent	13 (33.7%)	5 (38.5%)		17 (34%)	1 (50%)	
Perineural invasion						
Present	10 (25.6%)	4 (30.8%)	0.729	14 (28%)	-	1.000
Absent	29 (74.4%)	9 (69.2%)		36 (72%)	2 (100%)	
Lymphatic invasion						
Present	16 (41%)	6 (46.2%)	1.000	21 (42%)	1 (50%)	1.000
Absent	23 (59%)	7 (53.8%)		29 (58%)	1 (50%)	
Macroscopy						
Ulcerovegetative	32 (82.1%)	9 (69.2%)		40 (80%)	1 (50%)	
Ulcerative	3 (7.7%)	2 (15.4%)		4 (8%)	1 (50%)	
Annular	2 (5.1%)	1 (7.7%)		3 (6%)	-	
Vegetative	1 (2.6%)	1 (7.7%)		2 (4%)	-	
Polypoid	1 (2.6%)	-		1 (2%)	-	
Lymph node invasion						
Present	6 (41%)	6 (46.2%)	1.000	21 (42%)	1 (50%)	1.000
Absent	23 (59%)	7 (53.8%)		29 (58%)	1 (50%)	

Table 3. Comparison of the MLH1 and MSH2 staining with other histopathological findings in CRCs n (%) (Continue)

	MLH1+	MLH1–	P	MSH2+	MSH2–	P
N						
N0	23 (59%)	7 (53.8%)		29 (58%)	1 (50%)	
N1a	4 (10.3%)	–		4 (8%)	–	
N1b	5 (12.8%)	4 (30.8%)		8 (16%)	1 (50%)	
N2a	4 (10.3%)	1 (7.7%)		5 (10%)	–	
N2b	3 (7.7%)	1 (7.7%)		4 (8%)	–	
Tumor nodule						
Present	3 (7.7%)	2 (15.4%)	0.589	5 (10%)	–	1.000
Absent	36 (92.3%)	11 (84.6%)		45 (90%)	2 (100%)	
Stromal reaction						
Mild	10 (25.6%)	3 (23.1%)		13 (26%)	–	
Moderate	25 (64.1%)	10 (76.9%)		33 (66%)	2 (100%)	
Severe	4 (10.3%)	–		4 (8%)	–	
Intratumoral lymphocytic response						
None	7 (17.9%)	2 (15.4%)		9 (18%)	–	
Mild to moderate	28 (71.8%)	5 (38.5%)		32 (64%)	1 (50%)	
Marked	4 (10.3%)	6 (46.2%)		9 (18%)	1 (50%)	
Crohn-like response						
None	27 (69.2%)	5 (38.5%)	0.048	31 (62%)	1 (50%)	1.000
Mild to moderate	10 (25.6%)	5 (38.5%)		15 (30%)	–	
Marked	2 (5.1%)	3 (23.1%)		4 (8%)	1 (50%)	

CRCs: colorectal adenocarcinomas; MLH1: mutL homolog 1; MSH2: mutS homolog 2

Figure 4. MSH2 positively stained villous adenoma (x400)

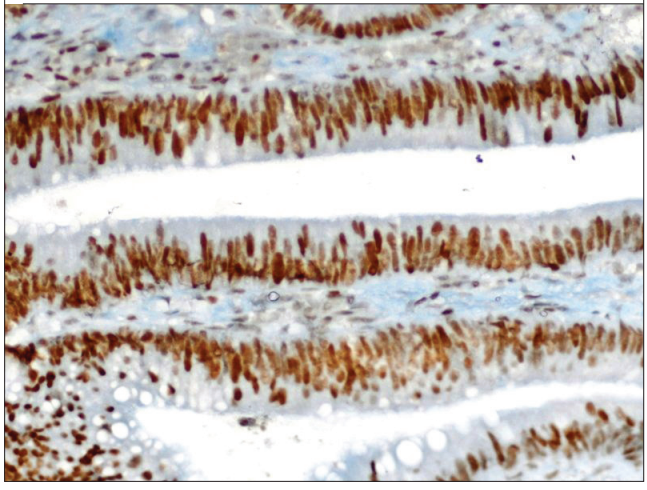
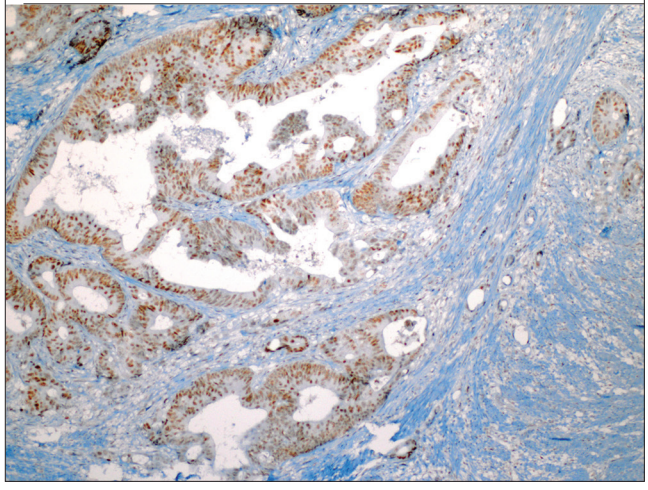


Figure 5. MLH1 positively stained adenocarcinoma, NOS (x100)



was no statistically significant difference between adenocarcinomas with mucinous component (negative staining in 20%) and signet-ring cell carcinomas (negative staining was not observed) in terms of the MSH2-negative staining (p>0.05) (Figure 13).

Figure 6. MLH1 negatively stained adenocarcinoma, NOS (x200)

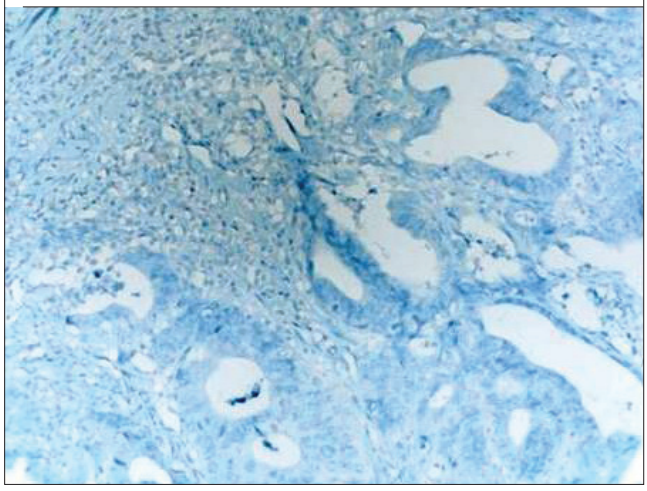


Figure 7. MSH2 positively stained adenocarcinoma (x100)

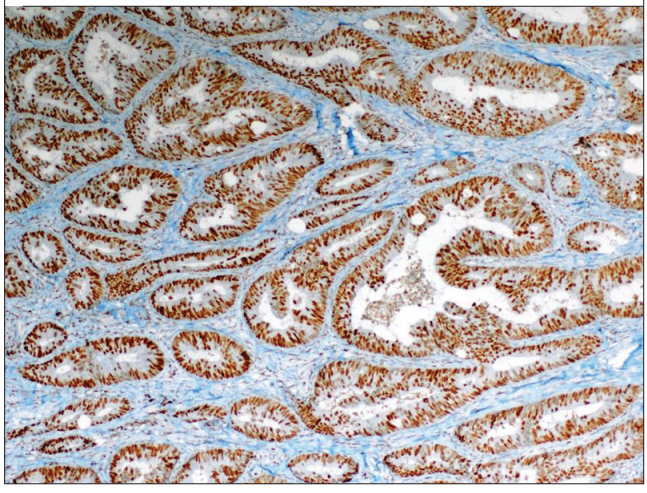
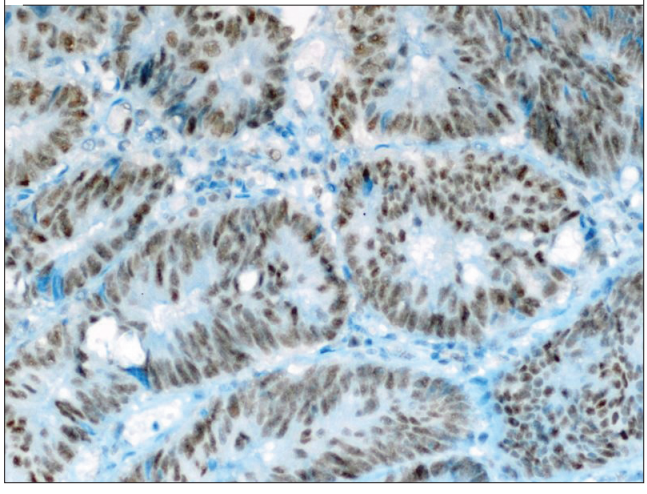


Figure 8. MLH1 positive adenocarcinoma with mucinous component (x400)



The intratumoral lymphocytic response was not observed in 15.4% (2 cases) of the MLH1-negative cases. The intratumoral lymphocytic response was mild to moderate in 38.5% (5 cases) and was significant in 46.2% (6 cases) of the MLH1-negative cases. Half of the MSH2-negative cases had mild-moderate intratumoral lymphocytic response, and half of them had marked intratumoral lymphocytic response. Crohn-like response was not seen in 38.5% (5 cases) of the MLH1-negative cases. Crohn-like response was observed in 38.5% (5 cases) of mild to moderate and was marked in 23.1% (3 cases) of the MLH1-negative cases. A significant statistical relationship was found between the presence of Crohn-like response and the MLH1-negative staining (p=0.048). A positive correlation was found between the MLH1-negative staining and intratumoral lymphocytic response (p=0.048, r=0.267) and Crohn-like response (p=0.031, r=0.292). A positive correlation was found between the MLH1-negative staining and intratumoral lymphocytic response (p=0.048, r=0.267) and Crohn-like response (p=0.031,

Table 4. Comparison of the MLH1 and MSH2 staining in CRCs between previous studies and our study n (%)

	Negative staining with MLH1	Negative staining with MSH2
Anderson et al.	177 (21%) (When negative staining is considered negative in one of the 4 mismatch repair proteins)	
Kenney et al.	13 (9.3%)	6 (4.3%)
Kumarasinghe et al.	12 (11%)	1 (1%)
Erdamar et al.	29 (39.2%)	5 (6.8%)
Lanza et al.	96 (13.4%)	18 (2.5%)
Jover et al.	11 (6.4%)	2 (1.16%)
Lindor et al.	228 (27.8%)	98 (11.9%)
Our study	13 (25%)	2 (3.8%)

CRCs: colorectal adenocarcinomas; MLH1: mutL homolog 1; MSH2: mutS homolog 2

Figure 9. MSH2 positive adenocarcinoma with mucinous component (x200)

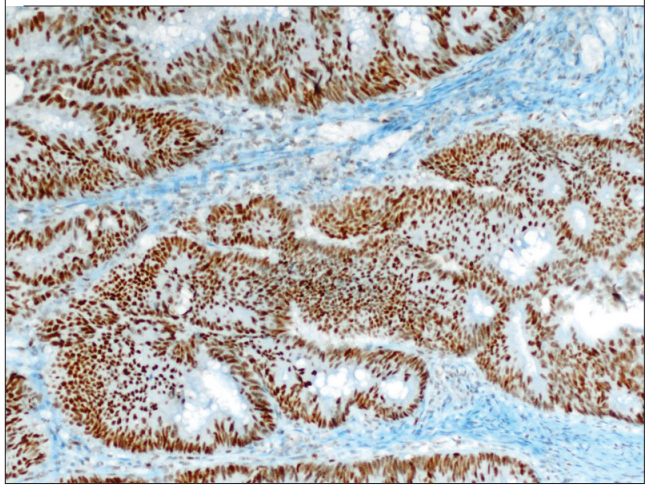


Figure 10. MLH1 positive mucinous adenocarcinoma (x400)

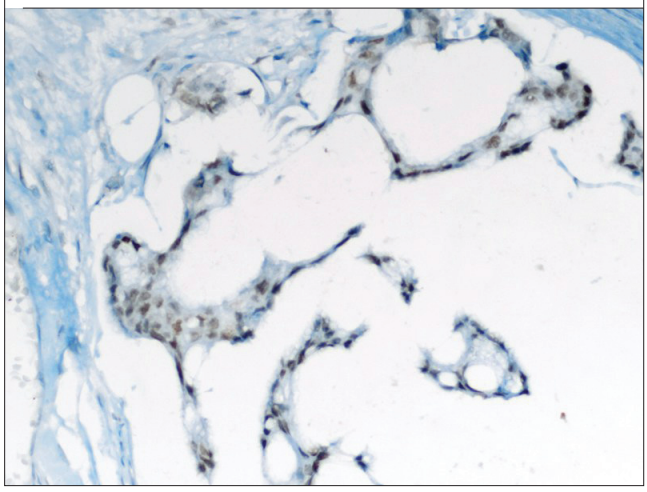


Figure 11. MSH2 positive mucinous adenocarcinoma (x200)

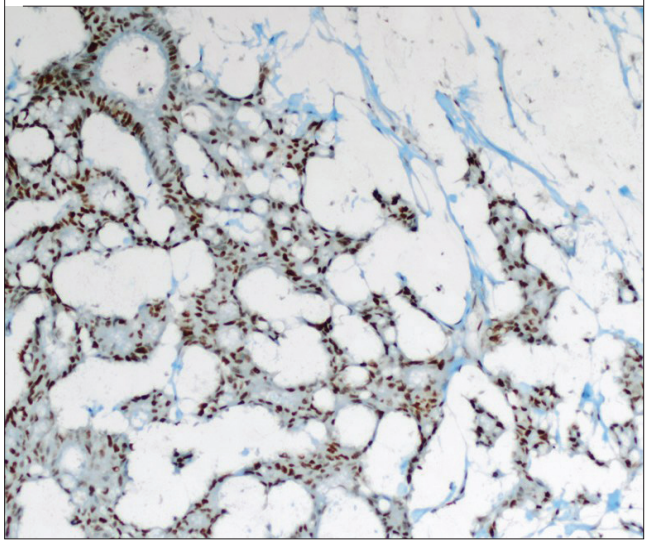


Figure 12. MLH1 positive signet-ring cell carcinoma (x400)

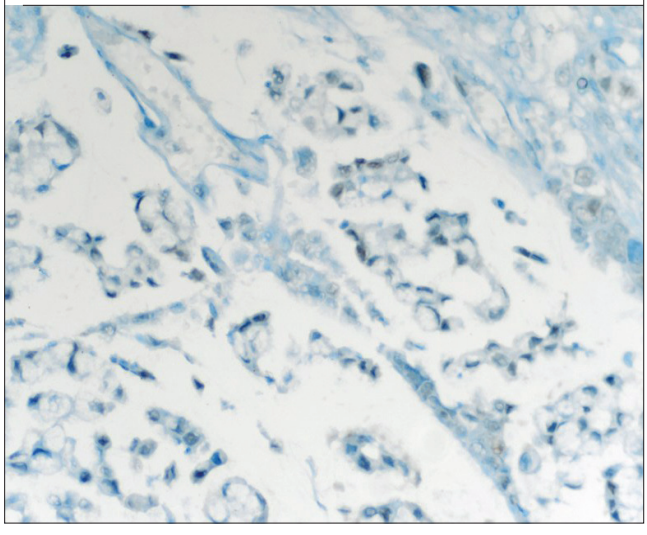


Figure 13. MSH2 positive signet-ring cell carcinoma (x400)

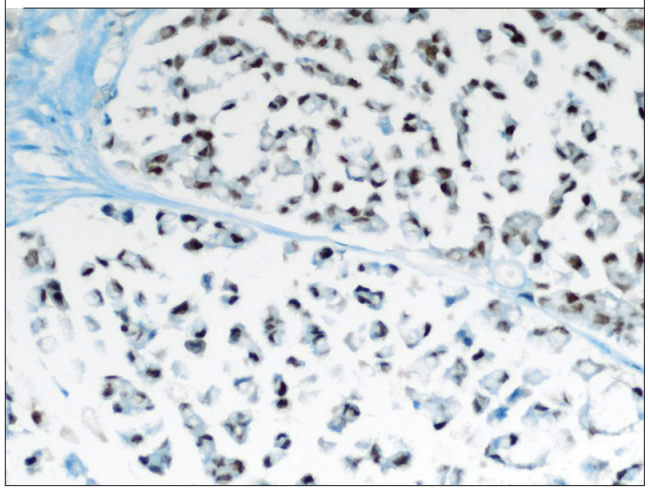


Figure 14. Positive correlation was found between the MLH1-negative staining and the intratumoral lymphocytic response

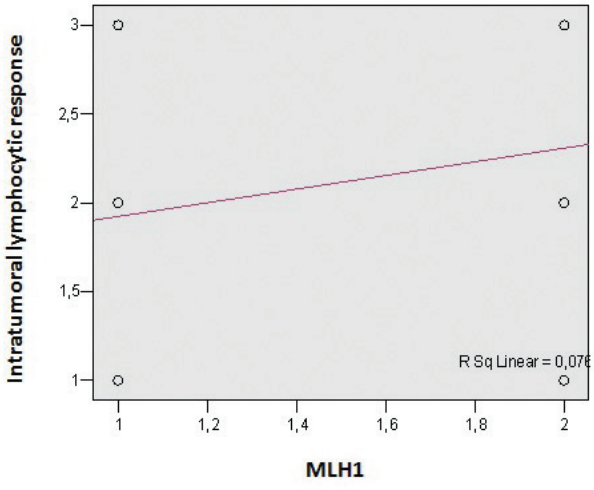


Figure 15. Positive correlation was found between the MLH1-negative staining and a Crohn-like response

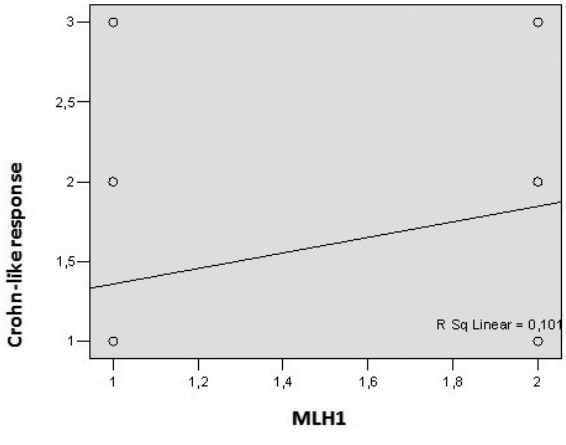


Figure 16. p53 staining in adenocarcinoma (G3) (x100)

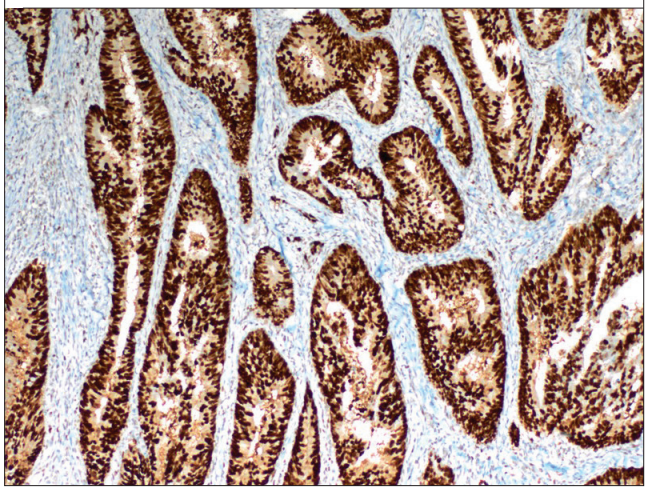
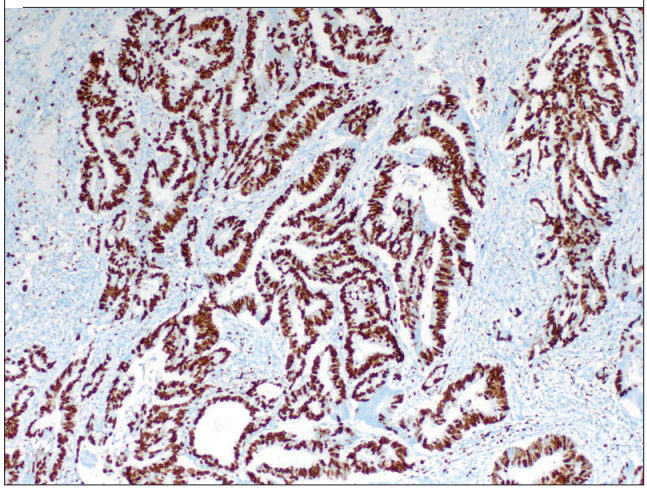


Figure 17. Ki-67 staining in adenocarcinoma (G3) (x100)



r=0.292) (Figures 14, 15). There was no statistically significant correlation between the intratumoral lymphocytic response/presence of the Crohn-like response and the MSH2-negative staining (p>0.05). No significant statistical relationship was found between the MLH1 and MSH2 and the p53 and Ki-67 staining (p>0.05) (Figures 16, 17).

DISCUSSION

Colorectal adenomas are common lesions, and their incidence increases especially over the age of 50 (1, 2). In our study, the mean age was 61 years. Of all the patients, only seven cases were under the age of 50 (14%). It is known that many factors such as gender, race, ethnicity, body mass index, lifestyle, nutrition, and drug use affect the incidence of adenomas (2). In our study, 42% of the cases were female, 58% were male, and the frequency of polyps was higher in males. In the literature, adenomas have a high incidence of distal colon localization under 60 years of age, while there was an increasing incidence of proximal colon localization over 60 years (2). In our study, when the cases were grouped as over and under 50 years old, no significant statistical relation was found between the groups and tumor localization (p>0.05).

The polyp diameter is the most important factor in determining the risk of carcinoma in an adenoma (2). When we compared the dysplasia grades in different sizes of polyps in our study, it was statistically determined that polyps with high-grade dysplasia (mean diameter 1.65 cm) had larger dimensions than polyps with low-grade dysplasia (mean diameter 0.76 cm) (p=0.005). In a study of 13,992 polypectomy cases, it was found that advanced histological features and cancer risk increased in polyps that were 1 cm in diameter or larger (8, 9). In our study, villous adenomas were found to be larger than tubulovillous and tubuler adenomas, and tubulovillous adenomas were found to be larger than tubuler adenomas. We found a statistically significant relationship between the histological type and size. There was no statistically significant relationship between gender and histological type in our study.

In our study, positive staining with MLH1 and MSH2 was detected in all of the adenomatous polyps. Basterra et al. (10) found negative staining with MLH1 and PMS2 in 6 cases, PMS2 in 2 cases, MSH6 in 1 case, and MSH2 in a study of 187 adenomatous polyps with high-grade dysplasia. The study by Kang et al. (11) included advanced adenomas in patients aged 40 years, and less and advanced adenomas in patients aged 50 years and over, as a control group. In this study, cases with the MLH1-negative staining were found in tubular morphology and in the young patient group. Balbinotti et al. (3) did not detect the loss of MLH1 and MSH2 in hyperplastic polyps in a study of 138 cases. They detected negative staining in 20% adenomas with MLH1 and 15.5% with MSH2. In this study, the MLH1 and MSH2 staining were not correlated with age, gender, the adenoma size, localization, histologic type of adenoma, and grade of dysplasia. Hawkins at al. (4) studied the polyps that were detected in CRC resection materials. In this study, hyperplastic polyps localized in the right colon were found to be associated with MSI colorectal carcinomas, and negative staining with MLH1 was detected in these polyps. So, it was suggested that serrated polyps could be the precursor lesions of MSI CRCs.

Sporadic colorectal carcinoma is more common in men than in women, and the incidence is increasing with age. CRC is rarely seen under 40 years of age without genetic predisposing factors or other predisposing factors such as inflammatory bowel disease. Macroscopically, most CRCs are described as ulcerovegetative (1). In our study, tumors were macroscopically 78% ulcerovegetative, 9.6% ulcerative, 5.8% annular, 3.8% vegetative, and 1.9% polypoid. In the literature, carcinomas of the proximal splenic flexure have been described as showing a more exophytic growth pattern, while carcinomas of the descending colon and rectum have been described as showing a more endophytic and annular growth pattern (1). In our study, ulcerovegetative appearance was the most common type, whereas we found annular tumors more frequently in the right colon and ulcerative tumors more frequently in the left colon.

The microsatellite instability status in sporadic colorectal carcinomas is accepted as a prognostic and predictive factor (1). High-frequency microsatellite instability (MSI-H) tumors have a better prognosis than low-frequency MSI/microsatellite stable

(MSI-L/MSS) ones. MSI-H tumors show a poor response to 5-fluorouracil and oxaliplatin, and MSI-H CRCs are sensitive to irinotecan treatment. MSI-H CRCs provide a better survival after adjuvant irinotecan treatment (8). Lanza et al. (12) found a better clinical outcome in the MLH1/MSH2-negative tumors in Stage 2 and 3 colorectal cancers in a colorectal carcinoma study involving 718 patients.

An indirect immunohistochemical MSI diagnosis can be given as a result of developing specific antibodies against the mismatch repair proteins, which are gene products of the mismatch repair system (1). A high level of agreement was found between the immunohistochemically detected MLH1 and MSH2 expressions and the MSI status determined by molecular methods (5-8). Lindor et al. (5) demonstrated 27.8% of negative staining with MLH1 and 11.9% negative staining with MSH2 in a study involving 1144 cases. In this study, the immunohistochemical detection of MSI with MLH1 and MSH2 was found to be 92.3% sensitive and 100% specific. Lanza et al. (12) reported 13.4% negative staining with MLH1 and 2.5% negative staining with MSH2 in a study including 718 cases, and the immunohistochemical staining of MLH1 and MSH2 was found to be consistent with the MSI status detected by molecular techniques. Jover et al. (7) showed negative staining with MLH1 in 6.4% of the cases and negative staining with MSH2 in 1.16% of the cases in their study. Erdamar et al. (13) detected 39.2% negative staining with MLH1 and 6.8% negative staining with MSH2 in a study including 77 cases. Kumarasinghe et al. (14) reported 11% negative staining with MLH1 and 1% negative staining with MSH2 in a total of 112 cases. Kenney et al. (15) reported 9.3% negative staining with MLH1 and 4.3% negative staining with MSH2 in a total of 141 cases. Andersen et al. (16) reported 21% negative staining (when negative staining was considered negative in at least one of the four mismatch repair proteins) in a study including 833 cases. In our study, we observed negative staining with MLH1 in 25% of all cases and with MSH2 in 3.8% of the cases. Similar to literature, the percentages of the MLH1-negative stained cases were higher than the MSH2-negative stained cases. The percentage of the MLH1-negative staining that we observed in our study was similar to the results by Lindor et al. (5). The percentage of the MSH2-negative staining that we detected in our study was similar to the results reported by Lanza et al. (12) and Kenney et al. (15) (Table 4).

Lanza et al. (12) found that the MLH1- and MSH2-negative cases were seen more frequently in women in their study. Erdamar et al. (13) reported that all of the MSH2-negative cases were found in women in their study. Similar to these studies, Andersen et al. (16) observed that MSI tumors were more frequent in women. We did not find a significant statistical relationship between gender and the MLH1 and MSH2 negativity in our study. Similar to our findings, Jover et al. (7) found no statistically significant relationship between the MLH1- and MSH2-negative staining and age and gender. Lanza et al. (12) found that the MLH1- and MSH2-negative cases were more common in subjects over 70 years of age when compared to positive cases. Erdamar et al. (13) found that the MSH2-negative cases were more common in subjects over 50 years of age. In the study by Andersen et al. (16), there was no significant relationship between the MSI status and age for CRC. We found no statistically significant relationship between the age groups and MLH1 and MSH2.

Andersen et al. (16) found that MSI tumors show the right colon localization more frequently. Lanza et al. (12) found that the MLH1- and MSH2-negative cases were located in the proximal colon. Erdamar et al. (13) reported that the majority of MSH2-negative tumors were located in the right colon. In our study, we found that the rate of MLH1-negative stained tumors was higher in the right colon than in the left colon.

Microsatellite instability tumors frequently exhibit an expansile growth pattern, with mucinous, medullary, or low differentiated morphology and marked host response (1, 16, 17). Most of the MSI tumors were detected in the histologic type of mucinous carcinoma (18). However, MSI was not detected in the majority of mucinous carcinomas (18). Mucinous carcinomas have been shown to be associated with good prognosis when showing MSI (1, 18).

In a study involving 323 sporadic CRC cases, Alexander et al. (19) reported that 15% of MSI-H tumors and 5% of MSS tumors were mucinous carcinomas. When the MSI-H and MSS tumor groups were compared in this study, tumors with mucinous component were found more frequently in the MSH-H group. Andersen et al. (16) reported MSI tumors were mostly mucinous, poorly differentiated, and of medullary carcinoma morphology. Erdamar et al. (13) observed no significant relationship between the MLH1/MSH2 expression and histologic type. Lanza et al. (12) found that the MLH1- and MSH2-negative cases showed a poorly differentiated morphology, mucinous and medullary histology, and marked Crohn-like response. Jover et al. (7) found a significant association between the tumor grade and the MLH1- and MSH2-negative staining; however, they did not find any statistically significant relationship between age, gender, tumor size, the pathological stage, mucin production, and MLH1/MSH2 staining. In our study, there was no statistically significant difference between adenocarcinoma, NOS, and adenocarcinoma with mucinous component in terms of negative staining with MLH1 and MSH2. However, similar to the literature, we found a statistically significant difference in our study between adenocarcinoma, NOS, and mucinous adenocarcinoma in terms of the MLH1-negative staining when compared with each other.

Lanza et al. (12) found that the MLH1- and MSH2-negative cases were poorly differentiated morphologically. Jover et al. (7) found a significant relationship between a high tumor grade and the MLH1- and MSH2-negative staining. Similarly, in our study, we found a statistically significant relationship between the histological grade and the MLH1 negativity. Signet-ring cell carcinomas with MSI show a better prognosis (1). Alexander et al. (19) found that 13% of the MSI-H tumors contained signet-ring cells. In the study by Andersen et al. (16), 33.3% of signet-ring cell carcinomas were detected as MSI. In our study, adenocarcinoma, NOS, and signet-ring cell carcinomas showed a statistically significant difference in terms of the MLH1-negative staining when compared with each other.

Tumor-infiltrating lymphocytes were found to be associated with MSI and good prognosis (18). Frequent MSI-H related morpholog-

ical findings, such as peritumoral lymphocytic response and tumor-infiltrating lymphocytes, are positive prognostic findings (1, 19). Alexander et al. (19) detected significant intraepithelial lymphocytosis in 21% of MSI-H tumors, while it was detected in only 3% of MSS tumors. In our study, we found a positive correlation between the MLH1-negative staining and intratumoral lymphocytic response in the CRCs. In half of the MSH2-negative cases, moderately marked intratumoral lymphocytic response were observed.

Crohn-like inflammation was found to be associated with good prognosis (18, 20, 21). Alexander et al. (19) observed no statistically significant difference in the presence of Crohn-like inflammatory responses in MSI-H and MSS tumors. Lanza et al. (12) found that the MLH1- and MSH2-negative cases showed a marked Crohn-like response. We found a statistically significant relationship between the Crohn-like response and MLH1-negative staining.

Andersen et al. (16) found less lymph node involvement in MSI tumors. We did not find any significant statistical relationship between the presence of lymphatic invasion/lymph node involvement and the MLH1/MSH2 staining.

CONCLUSION

We conclude that routine immunohistochemical staining for MLH1 and MSH2 may be useful in the detection of MSI in tumors in the presence of mucinous and poorly differentiated morphology, tumor-infiltrating lymphocytes, and Crohn-like inflammatory response in the histopathological examination of colorectal tumors.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Recep Tayyip Erdoğan University School of Medicine (Approval date: 24 April 2016; number: 2016/18).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - R.Y., R.B.; Design - R.Y., R.B.; Supervision - R.Y., R.B.; Resources - R.Y., R.B., R.A.A., A.P.; Materials - R.Y., R.B., R.A.A., A.P.; Data Collection and/or Processing - R.B., R.Y.; Analysis and/or Interpretation - R.Y., R.B.; Literature Search - R.Y., R.B.; Writing Manuscript - R.Y., R.B.; Critical Review - R.Y., R.B.

Conflict of Interests: The authors have no conflicts of interest to declare.

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Original Research

The Frequency of Celiac Disease in Turkish Children with Cystic Fibrosis

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ABSTRACT

Objective: The aim of the present study was to investigate the frequency of celiac disease (CD) in children with cystic fibrosis (CF).

Methods: This prospective study was conducted from October 2015 to March 2017. A total of 71 patients with CF and 73 age- and sex-matched healthy children were included in the study. All groups were evaluated for CD with regard to clinical and laboratory findings. First, total IgA and tissue transglutaminase IgA (tTG IgA) levels were measured. Anti-endomysium IgA antibodies (EMAs) were analyzed for those patients with positive tTG IgA. Gastroduodenoscopy was performed to patients with both positive tTG and EMA IgA.

Results: Only 8 (11.2%) patients had tTG IgA positivity, whereas 4 (5.6%) patients had positive EMA. The pathological results were consistent with the Marsh 2 classification score in two patients. In addition to that, HLA-DQ2 is present in those two patients. Those patients were accepted as potential CD. Two patients who were thought to have potential CD were reassessed after 1 year, and celiac screening tests were detected as positive again.

Conclusion: Only 2 (2.8%) of 71 patients with CF were diagnosed with potential CD. Our study results showed that there might be an association between CD and CF.

Keywords: Celiac disease, cystic fibrosis, small intestine biopsy

INTRODUCTION

Cystic fibrosis (CF) is the most common autosomal recessive disorder associated with a deficit in the cystic fibrosis transmembrane regulator (CFTR) gene localized on chromosome 7 and is life-threatening among Caucasians (1). The global incidence of CF is 1 in 2500 newborns (2).

Celiac disease (CD) is an immune-mediated systemic disease characterized by intestinal villous damage at various levels, triggered by gluten intake in genetically susceptible individuals (3).

The prevalence of CD is estimated to be 0.5%-1% in different regions worldwide (4). CD has two peaks that are between 1 and 2 years old and 30 years old (5).

Cystic fibrosis was first described in 1938 and has been considered a separate disease from CD since then. A patient with CD and CF was reported for the first time in 1969, and after that, sporadic cases were reported (1).

Weight loss, steatorrhea, and diarrhea are associated with intestinal malabsorption in both diseases; thus, it is difficult to diagnose CD in patients with CF (6). It has been recommended that screening of CD should be performed in communities with a high prevalence of CD and those with CF particularly with malabsorption that does not respond to standard treatments, those with pausing in physical development, and those with CF with comorbidity of autoimmune disease (1, 6). There is no consensus on the screening of CD in children with CF.

There are very few studies investigating the prevalence of CD in patients with CF (6-9). To our knowledge, there is no study that investigates the frequency of CD in children with CF in our country. For this reason, the aim of the present study was to investigate the frequency of CD in children with CF.

METHODS

The study was conducted prospectively at the Outpatient Clinics of Pediatric Pulmonology and Gastroenterology between October 2015 and March 2017. A total of 71 patients were included

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in the study. Patients with incomplete information in their files at the time of diagnosis, patients with coincidental disease, and patients who refused to participate in the study were excluded from the study. Only three patients refused to participate in the study. Patients who were followed up with a diagnosis of CF, received gluten, and wanted to participate in the study voluntarily were included in the study. A total of 73 age- and sex-matched healthy children were included in the study as the control group.

The study protocol was approved by the local ethics committee (313608, October 6, 2015). Informed written consent was obtained from the patients, the healthy controls, and their parents before participation in the study. Those who refused to participate in the study and those who did not receive gluten were not included in the study.

Cystic fibrosis was diagnosed according to the generally accepted criteria (10). All groups were evaluated for clinical and laboratory findings with regard to CD. Venous blood samples were obtained from both groups. Each sample was divided into aliquots, and samples were stored at –80 °C until analysis.

Total IgA tests (Roche Diagnostics GmbH, Mannheim, Germany) by immunoturbidimetric method and tissue transglutaminase (tTG) IgA tests (catalog no. 3503; Aesku Diagnostics GmbH, Wendelsheim, Germany) by ELISA method were measured at the Central Biochemistry Laboratory of Cerrahpaia Medical Faculty. The tTG IgG test was planned for patients with IgA deficiency. The cut-off value of tTG antibody was 12 U/mL. Anti-endomysium IgA antibody (EMA IgA) test (Inova Diagnostics, Inc., Lübeck, Germany) by IFA method was analyzed in patients with positive tTG IgA in the Düzen Laboratories Group in İstanbul, Turkey. Gastroduodenoscopy was performed to the patients with both positive tTG and EMA IgA for definitive diagnosis.

Biopsy Procedures

Gastroduodenoscopy was performed to the patients with both tTG and EMA positivity. One biopsy from the duodenal bulb and four biopsies from the duodenum were obtained. Biopsies were evaluated by the same experienced pathologist according to the Marsh classification score (11).

Statistical Analysis

Statistical Package for the Social Sciences for Windows, version 17.0 software (SPSS Inc.; Chicago, IL, USA) was used for statistical analysis. Descriptive statistics were expressed as frequency, percentage, mean ± standard deviation, and median (interquartile range). Independent samples t-test was used for nominal data with normal distribution. Mann-Whitney U test was used for patients with non-normally distributed variables, and chi-square test was used to compare the relationship between categorical variables. A p value <0.05 was considered statistically significant.

RESULTS

Overall, 71 patients were included in the study. The mean age and weight of the patients were 9.94±5.55 years and 29.32±15.03 kg, respectively. Of 71 patients, 36 (50.7%) were female, and 35 (49.3%) were male. There were 37 (50.7%) girls and 36 (49.3%) boys in the control group. The mean age of the control group was 9.67±5.36 years. When the patient and control groups were compared with regard to age, sex, height, and weight, there was no significant difference between the groups (p>0.05) (Table 1).

Of 71 patients, 56 (78.9%) have gastrointestinal symptoms, such as chronic diarrhea, steatorrhea, and weight loss. In addition, 15 (21.1%) have anemia for extraintestinal symptoms.

tTG IgA positivity was detected in only 8 (11.2%) patients, then EMA was measured, and 4 (5.6%) patients had positive results (Table 2). Gastroduodenoscopy was performed to those patients.

Table 1. Demographic characteristics and laboratory findings of the patient and control groups

	Patient group (n=71)	Control group (n=73)	p
Age (years)*	10.75	10.00	0.873
Height (cm)*	134.00	137.00	0.440
Weight (kg)*	27.40	37.00	0.090
Hemoglobin (mg/dL)**	12.61±1.07	12.78±1.48	0.434
MCV**	80.95±5.04	79.39±4.87	0.060
Plt (/mm^3)*	319.00	287.00	0.066
tTG IgA (U/mL)*	1.70	0.90	0.001
Total IgA (mg/dL)*	141.00	133.00	0.414
Age at diagnosis (months)*	6.00	–	
Sweat chloride level (mEq/L)**	97.11±21.8	–	

Plt: thrombocytes; MCV: mean corpuscular volume; tTG: tissue transglutaminase
*Data are presented as median (interquartile range)
**Data are presented as mean±standard deviation

Table 2. Data of patients with positive tissue transglutaminase antibody

Patient no.	tTG IgA (U/mL)	Total IgA (mg/dL)	EMA IgA	Marsh classification score
1	300	84	+	2
2	47.1	147	+	2
3	72.9	241	+	0
4	15	69.3	+	0
5	31.4	184	–	–
6	32.6	92	–	–
7	26.5	187	–	–
8	33.8	210	–	–

tTG: tissue transglutaminase; EMA: anti-endomysium antibody

Table 3. Laboratory data of patients with potential celiac disease

Patient no.	tTG IgA (U/mL)	Total IgA (mg/dL)	EMA IgA	Marsh classification score
1	300	84	+	1
2*	32	147	+	–

tTG: tissue transglutaminase; EMA: anti–endomysium antibody
*Gastroduodenoscopy could not be performed due to chronic infection

One biopsy from the duodenal bulb and four biopsies from the duodenum were obtained. The endoscopic appearance of two patients has normal mucosal appearance of the bulbus and scalloping of the duodenum. The pathological results were consistent with the Marsh 2 classification score in those two patients, and HLA-DQ2 typing was also positive. Those patients were accepted as potential CD. In addition, they have typical gastrointestinal symptoms, such as chronic diarrhea and steatorrhea; thus, they are considered as typical CD.

The other two patients have normal mucosal appearance of the bulbus and duodenum, and their pathological results were compatible with the Marsh 0 classification score. Two patients who were thought to have potential CD were reassessed after 1 year, and celiac screening tests were detected as positive again. Gastroduodenoscopy could not be performed to one patient due to chronic pulmonary infection. In the other patient, the pathological result was consistent with the Marsh 1 classification score (Table 3).

In the control group, 3 (4.1%) children had tTG IgA positivity, but EMA positivity was not detected in any of them.

DISCUSSION

The CFTR gene associated with CF disease was identified in 1989. The most common CFTR defect is delta F508 mutation, which is present in approximately 70% of patients with CF (2). Currently, more than 1500 mutations have been identified (12). Different CFTR mutations result in different disease phenotypes. Some mutations may have less or no effect on the CFTR function or may cause mild forms of the disease (13).

The prevalence of CD has increased dramatically in the last 20 years due to the use of serological tests. Previous studies reported that its prevalence increased four times in the United States, whereas it increased two times in Finland (14, 15). Since only 10% of patients are symptomatic, the majority of asymptomatic patients remain undiagnosed despite screening of the high-risk populations (5, 16).

Celiac disease is a lifelong disorder and is associated with increased morbidity and mortality if left untreated (17). CD complications are predominantly in adults and include refractory CD, decreased fertility in women, cancers, and other autoimmune diseases. Compared with the general population, the risk of developing cancer is twice as high (18). With a gluten-free diet, the risk of developing complications is likely reduced (19). Therefore, it is very important to diagnose CD early and to start treatment.

Most of the symptoms of CD can be seen as gastrointestinal system findings of CF. Furthermore, it is difficult to distinguish between these two conditions in patients with CF, since clinical findings of iron deficiency anemia and lack of fat-soluble vitamins are equally seen among people with and without CD (6, 7). Mucosal changes in the small intestine and its associated malabsorption may worsen nutritional status and affect survival duration. Thus, diagnosing CD in coexisting patients with CF may play an important role in treatment efficacy (7).

Some hypotheses have been proposed to explain the coexistence of these two diseases. In patients with CF, the small bowel mucosa may have greater contact with the gluten protein due to incomplete digestion and pancreatic insufficiency. This can play an important role in comorbidity. However, malnutrition can also cause some additional mucosal damage. In patients with malabsorption, feeding with a high-energy diet results in more antigen burden, and gluten peptides may pass more easily to the epidermis and lead to the development of CD (7, 20).

Venuta et al. (21) reported a case report with an association of CD and CF, and they had opted for 15 cases of the coexistence of CD and CF as reported in the literature until 1999.

Previous studies have reported that the incidence of CD among children with CF is between 0.4% and 2.6% (6-9).

In a multicenter study, the prevalence of CD was found to be 1.2% (1/83) in 790 Scandinavian patients with CF (6). In this study, six patients were already diagnosed with CD, whereas four were recently diagnosed with CD. In addition, serological tests were positive in two patients, but normal histology of duodenal biopsy was detected. These patients were scheduled for follow-up because of suspected potential CD. Based on the prevalence studies conducted previously, it has been estimated that the prevalence of CD among Scandinavian patients with CF was 2-3 times higher than that of the general population. CD was diagnosed in three patients before the diagnosis of CF, whereas it was synchronously diagnosed with CF in two patients. According to clinical findings, it was difficult to distinguish between these two conditions, and iron deficiency and lack of fat-soluble vitamins were found to have equal prevalence in these patients. In this study, it has been recommended that screening should be performed for children >9 months with CF or those receiving gluten for >3 months in populations with a high prevalence of CF, such as Sweden (6).

In a study including 230 patients with CF in Poland, the prevalence of CD was found to be 2.6%. tTG IgA positivity was detected in 11 patients, and EMA IgA positivity was detected in six of those patients. Gastroduodenoscopy was performed to five of them, four were diagnosed with CD at that moment, and two were previously diagnosed. The incidence of CD was five times higher in patients with CF than in healthy populations. In addition, tTG positivity and EMA negativity were found in four patients, and gastroduodenoscopy was performed for those four patients, and the pathological results were detected as normal. Moreover, those patients were planned to be followed up for a long time, as

they might have latent CD (7). It has been suggested that CF as a risk factor should be examined as a consequence of this study, and it is thought that CF has a tendency to cause CD.

Abdominal pain was detected in 11% of the 500 patients with CF, and 2 (0.4%) patients were diagnosed with CD (8). It has been stated that the most common cause of admission to the hospital in older children with CF might be abdominal pain.

In the study conducted by Valleta and Mastella (9), the incidence of CD was found to be 0.4% in 1100 patients with CF and reported to have a higher incidence of CD in a population with CF than that in a healthy population.

The current approach to CD has changed with the development of highly sensitive and specific serological tests. Both EMA IgA and tTG IgA tests have been shown to be highly sensitive for CD (22). The European Association of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends total IgA and tTG IgA tests for initial screening of CD. It has been suggested that EMA IgA test should be performed in patients with tTG IgA positivity. If this test is found to be positive, small bowel biopsy should be performed (3). In patients with IgA deficiency, tTG IgG or EMA IgG may help in the decision for biopsy (16). EMA and tTG IgA tests have >95% sensitivity and specificity when used together (23).

Our study was based on the updated ESPGHAN guideline. tTG IgA positivity was detected in 8 (11.2%) patients, then the levels of EMA IgA were measured for these patients, and positive results were detected in 4 (5.6%) patients (Table 2). Gastroduodenoscopy was performed in those patients. The pathological results were compatible with the Marsh 2 classification score in two patients who had also positive HLA-DQ2 tests. Potential CD was thought in those two patients, and it was planned that those patients should be followed up serologically and clinically (24,25). In the other two patients, the pathological results were detected as normal. Two patients who were thought to have potential CD were reassessed after 1 year, and the celiac serological tests were found to be positive again. The first patient could not undergo endoscopy due to chronic infection. In the second patient who underwent endoscopy, the pathological result was compatible with the Marsh 1 classification score. In the latter patient, the patient did not have a gluten-free diet, and the pathological result of the second intestinal biopsy may be associated with the patchy distribution of villous atrophy of CD. In addition, the first patient who could not undergo endoscopy did not have a gluten-free diet. If we had performed gastroduodenoscopy to the first patient, we could detect the Marsh 2 classification score. Therefore, in the present study, the frequency of potential CD was 2/71.

As a limitation, the number of cases may be small due to the fact that our study was a single-center study, but only three follow-up patients did not accept to participate voluntarily. Therefore, the impact of our study may be weak, but we still consider that the present study is important for our country, as to the best of our knowledge, this is the first study on this issue.

In a study conducted in healthy Turkish children aged between 6 and 17 years in our country, the prevalence of CD was detected as 0.47% (26). According to our study results, the frequency of potential CD was found to be 2.8% in patients with CF. We detected that CD was seen approximately six times more in the CF population than in healthy children.

In conclusion, despite the small number of cases, we suggest that all children with CF should be screened for CD. Multicenter studies with more children with CF are needed to provide more precise evaluation.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of İstanbul University-Cerrahpaşa, Cerrahpaşa School of Medicine (313608, October 6, 2015).

Informed Consent: Informed written consent was obtained from the patients, the healthy controls, and their parents before participation in the study.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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Original Research

Can the Ratio of Calcium to Albumin Predict the Severity of Aortic Stenosis?

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ABSTRACT

Objective: Aortic sclerosis is observed in 25% of the elderly population, and 2.5% of these patients have severe aortic stenosis (AS). Numerous studies have reported a relationship between the serum calcium or albumin levels and AS. The present study investigated the relationship between the calcium toalbumin ratio (CAR) and AS.

Methods: Our study included 185 patients and 108 subjects as the control group. A routine transthoracic echocardiographic evaluation and laboratory examinations were performed inall participants. The corrected serum calcium levels were calculated using the most commonly used formula: corrected calcium=measured total calcium (mg/dL) + 0.8 (4.0−serum albumin [g/dL]).

Results: The serum C-reactive protein CRP, calcium, and corrected calcium levels were significantly different between the study groups ($p<0.05$), and the albumin levels were significantly decreased parallel withthe AS severity ($p<0.001$). Also, we detected a negative correlation between the albumin and corrected calcium levels and the EuroSCORE. CAR and corrected calcium to albumin ratio (cCAR) were significantly higher in the AS group, as expected ($p<0.01$). In the logistic regression analysis, albumin, CRP, low-density lipoprotein LDL, theCAR, and cCAR levels were found to be significantly and independently associated with the presence of AS ($p<0.05$). Moreover, in a regression analysis in the subgroup of AS only, albumin, the cCAR, and CAR were independently associated with the presence of very severe AS.

Conclusion: Our study showed an important relationship between the CAR and AS. Therefore, in clinical practice, this simple, inexpensive, and practical method may predict the severity of AS.

Keywords: Albumin, aortic stenosis, calcium, calcium to albumin ratio

INTRODUCTION

The best-known pathologies underlying aortic stenosis (AS) include aortic valve thickening and sclerosis, followed by progressive calcification causing obstruction. Aortic stenosis is observed in 25% of the elderly population (1), and changes on the cusp share similarities with the structure of atherosclerotic plaque (2). Approximately 2.5% of these patientsface severe AS, along with progressive calcification (3). Within this progressive process, the changes in the valve include bone cells in the osteoblastic phenotype, and in advanced AS cases, bone structures showing a lamellar structure (4, 5).

A previous study found that low serum calcium levels wereassociated with increasedcalcium hydroxyapatite deposition in native aortic valves in patients with severe calcific AS (6). On the other hand, another study demonstrated that serum calcium levels werehigher in patients with AS among subjects with normal renal function who did not have apparent atherosclerosis (7). In another study, while no significant relationship was detected between the severity of AS and serum calcium levels, it was reported that the increase in serum phosphate levels and the calcium-phosphate product levels have a negative relationship with the aortic valve area (AVA) (8). It has been previously shown that in patients with AS who underwent a transcatheter aortic valve implantation (TAVI) procedure, a low serum albumin level is an important prognostic factor for post-procedure mortality (9). Being an important marker of fragility, the serum albumin levels have been demonstrated to be lower in patients with AS, which is seen at an advanced age and in fragile patients (10). Additionally, inflammation has been demonstrated to play an active role in the progression of aortic sclerosis and calcific AS (11, 12). Also, albumin is known as a negative acute-phase reactant of which the blood level decreases during inflammation (13). Moreover, the most important transporter protein of calcium ions in the blood is albumin (14).

Our study explored the relationship of calcific AS with serum albumin, calcium, albumin-corrected calcium levels, and espe-

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cially the ratio of two closely interacting molecules, calcium and albumin.

METHODS

Our study included 185 patients admitted to our clinic between January 2014 and January 2017, diagnosed with severe AS, and 108 subjects who were admitted to our cardiology clinic for examination purposes and for whom no obstructive coronary artery disease (CAD) was detected after non-invasive tests, including the exercise test, myocardial perfusion scintigraphy, and computerized tomographic angiography, and no valvular pathology was detected byechocardiography. Patients with ahistory of acute coronary syndrome within the pastmonth; those with active malignancy;those who usedimmunosuppressive, steroid, or diuretic agents on regular basis, or external Vitamin D or calcium supplements;those who suffered fromchronic renal disease or renal disease requiring hemodialysis;those with aclinical presentation of infective endocarditis;those who suffered from anacute or chronic connective tissue disease;those who hadAS but with no available previous medical records; thosewho hadprimary hyperparathyroidism or inadequately treated thyroid issues;and those who hadaclinical presentation of Stage 3-4 cardiac failure were excluded from the study. Our study was designed in accordance with the Declaration of Helsinki, and the local ethical committee of Yıldırım Beyazıt University approved the study protocol (Date: 05.03.2018, No: 56). All patients were informed about the aims and the protocol of the study, and written informed consent was obtained.

Transthoracic Echocardiographic Evaluation

All patients underwent the echocardiographical examination, and the left ventricular ejection fraction was calculated using the modified Simpson method. It was performed using a lE33 echocardiography system (Philips Medical Systems, Eindhoven, The Netherlands) with a 3.5 MHz transducer by two experienced operators. The parasternal short- and long-axis, apical four-chamber, and subcostal four-chamber views were used as standard echocardiography. The aortic jet velocity was calculated by Doppler echocardiography. The transvalvular pressure gradient was determined by the Bernoulli formula, and AVA was calculated by the continuity equation. AS was defined as mild if the mean systolic transaortic gradient was <25 mmHg or the jet velocity was <3.0 m/s, moderate if the mean systolic transaortic gradient was 25-40 mmHg or jet velocity was 3.0-4.0 m/s, severe if the mean systolic transaortic gradient was >40 mmHg or jet velocity was >4.0 m/s, and very severe if the jet velocity wasgreater than 5.0 m/sn (15, 16).

Routine Laboratory Examinations

After 12 h of the fasting period, the blood for routine hematologic and biochemical tests was collected. The serum levels of fasting plasma glucose, lipid parameters, creatinine, and the hematological values were determined using the standard methods. The serum albumin and calcium levels were calculated using the COBAS INTEGRA Albumin Gen. 2/cobas c systems (Roche Diagnostics Corporation; Manheim, Germany). We used a reference range 3.5-5.2 g/dL and 8.8-10.2 mg/dL for albumin and calcium tests, respectively. The corrected serum calcium levels were calculated using the most commonly used formula in clinical practice, if the serum albumin level was <4 mg/dL: corrected calcium=measured total calcium (mg/dL) + 0.8 (4.0−serum albumin [g/dL]) (17).

Statistical Analysis

The data collected during the research were analyzed using the Statistical Package for the Social Sciences 15.0 statistical package program (SPSS Inc.; Chicago, IL, USA). Descriptive statistics were depicted as the mean±standard deviation or median (inter-quartile range) for continuous variables, and as the number of cases (n) and percentages (%) for categorical variables. The normality distribution was evaluated using the Kolmogorov-Smirnov test. Baseline characteristics were compared with the independent sample t-test, Mann-Whitney U test, chi-square test, or Fisher's exact test (wherever applicable). Pearson and Spearman's correlation test was used to assess the correlation between calcium, albumin, calcium/albumin ratio, and the mean systolic transaortic gradient and AVA. A logistic regression analysis was used to examine the association between AS, severe AS, and other variables. Variables with a p-value of <0.1 in a univariate logistic regression analysis were included in a multivariate logistic regression model. A p-value <0.05 was considered to be statistically significant.

RESULTS

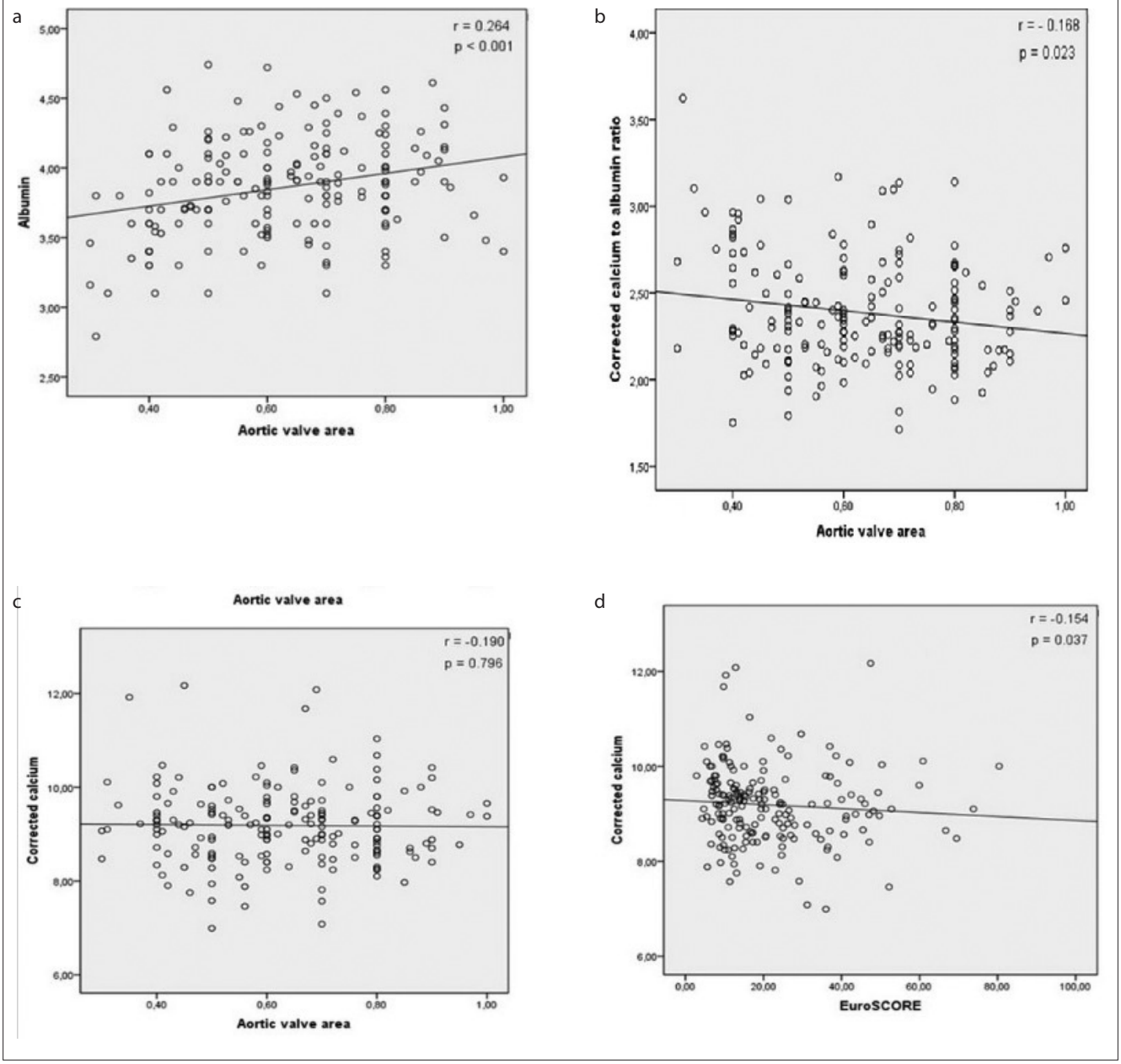
Clinical and demographic characteristics of the study groups are shown in Table 1. Hypertension and diabetes mellitus rates were higher, as expected, in the AS group (n=185) than in the control group (n=108), but there was no statistically significant difference in the subgroup between AS (n=145) and very severe AS group (n=40) (Table 2). The serum C-reactive protein (CRP), calcium, and corrected calcium levels were significantly different between the study groups (p<0.05), but only the serum CRP levels were still found to be significantly higher in the subgroup of AS (p=0.039). In addition, thealbumin level was significantly decreased parallel withthe severity of AS (4.49±0.51 in the control group, 3.90±0.34in the AS group, and 3.69±0.31in the very severe AS group; p<0.001). There was a significant positive correlation between albumin and AVA (r=0.264, p<0.001). Moreover, we did not observe a positive or negative correlation between AVA and neither calcium nor corrected calcium (respectively, r=0.05, p=0.485; and r=−0.190, p=0.796).Also, we detected a negative, but weak correlation between the albumin and corrected calcium levels and the EuroSCORE (Figure 1).

In addition, the calcium to albumin ratio (CAR) and correctedcalcium to albumin ratio (cCAR) were significantly higher in the AS group, as expected (p<0.01). Although the CAR and cCAR were detected as numerically higher in the very severe AS grup than the AS group, they were statistically insignificant (respectively, p-value 0.548 and 0.341). Also, the cCAR was positively correlated with the CRP level (r=0.197, p=0.019) and negatively correlated with AVA (r=−0.168, p=0.023) (Figure 2).

To determine the possible confounding factors for AS, a logistic regression analysis was performed. In the logistic regression analysis,the albumin, CRP, low-density lipoprotein (LDL), CAR, and

Figure 1. a-d. Correlation analysis of (a) serum albumin levels and AVA, (b) serum albumin levels and EuroSCORE, (c) serum calcium levels and AVA, and (d) serum calcium levels and EuroSCORE

AVA: aortic valve area



cCAR were found to be significantly and independently associated with the presence of AS (p<0.05). Moreover, the regression analysis in the AS only subgroup showed that albumin (odds ratio OR, 6.134; 95% confidence interval CI, 1.967-19.136; p=0.001) and cCAR (OR,4.613; 95% CI,0.930-22.876; p=0.0470) and CAR (OR,10.342; 95% CI, 1.252-24.296; p=0.030) were independently associated with the presence of very severe AS (Table 3).

DISCUSSION

The most important finding of our study is that AS is independently correlated with the CAR and albumin levels. To the best of our knowledge, this is the first large-scale study to investigate the association between the CAR and AS. We demonstrated that the CAR was significantly higher in patients with severe AS than in control subjects. Also, the CAR had a significantly positive correlation with CRP and negative correlation with AVA. Besides, we showed that the lower albumin levels and a higher CAR were also independently associated with the presence of very severe AS.

Aortic stenosis is a chronic disease thatshares similarities with atherosclerotic CAD, such as mechanical stress, chronic inflammation, calcification, and lipid deposition on valvular leaflets (2, 18). Being a commonly used and an importantmarkerof inflammation, it has been demonstrated that CRP levels are increased

Table 1. Baseline characteristics and laboratory parameters of the study groups

Variables	Control Group (n=108)	Aortic Stenosis (n=185)	p
Age, (years) (mean±std)	77.87±7.53	76.37±6.02	0.079
Hypertension, n (%)	45 (41.6)	143 (77.3)	<0.001
Diabetes mellitus, n (%)	10 (9.2)	59 (31.8)	<0.001
Creatinine, mg/dL, mean±std	0.84±0.21	0.97±0.4	0.06
LDL-C, mg/dL, (median-IQR)	119 (37-236)	100 (31-358)	<0.001
HDL-C, mg/dL, (median-IQR)	45 (23-80)	46 (12-103)	0.842
Triglyceride, mg/dL, mean±std	141 (34-584)	102 (30-511)	<0.001
CRP, (median-IQR)	3.61 (0.8-16)	4.62 (1.1-37)	0.042
Calcium (mg/dL) (median-IQR)	9.0 (6.91-12.17)	9.52(8.30-10.40)	<0.001
cCalcium (mg/dL) (median-IQR)	9.19 (6.9-12.17)	9.52 (8.5-10.40)	<0.001
Albumin (gr/dL) (mean±std)	4.49±0.51	3.88±0.35	<0.001
Calcium/albumin ratio	2.12±0.18	2.33±0.26	<0.001
Corrected calcium/albumin ratio	2.12±0.19	2.38±0.31	<0.001
LVEF, %, (mean±std)	61.46±4.64	53.07±14.30	<0.001
Maximum gradient,mmHg	-	82 (36-187)	-
Mean gradient, mmHg	-	49 (20-114)	-
Aortic valve area (cm^2), (median-IQR)	-	0.64 (0.30-1.0)	-
Logistic EuroSCORE (%)	-	16.54 (2.86-60)	-
Society of Thoracic Surgeons score (%)	-	5.7 (1.20-31.2)	-

CAD: coronary artery disease; cCalcium: corrected calcium; CRP: C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; LVEF: left ventricular ejection fraction; WBC: white blood cell

Figure 2. a, b. Correlation analysis of (a) cCAR and CRP, and (b) cCAR and AVA
AVA: aortic valve area; CRP: C-reactive protein; cCAR; corrected calcium to albumin artio

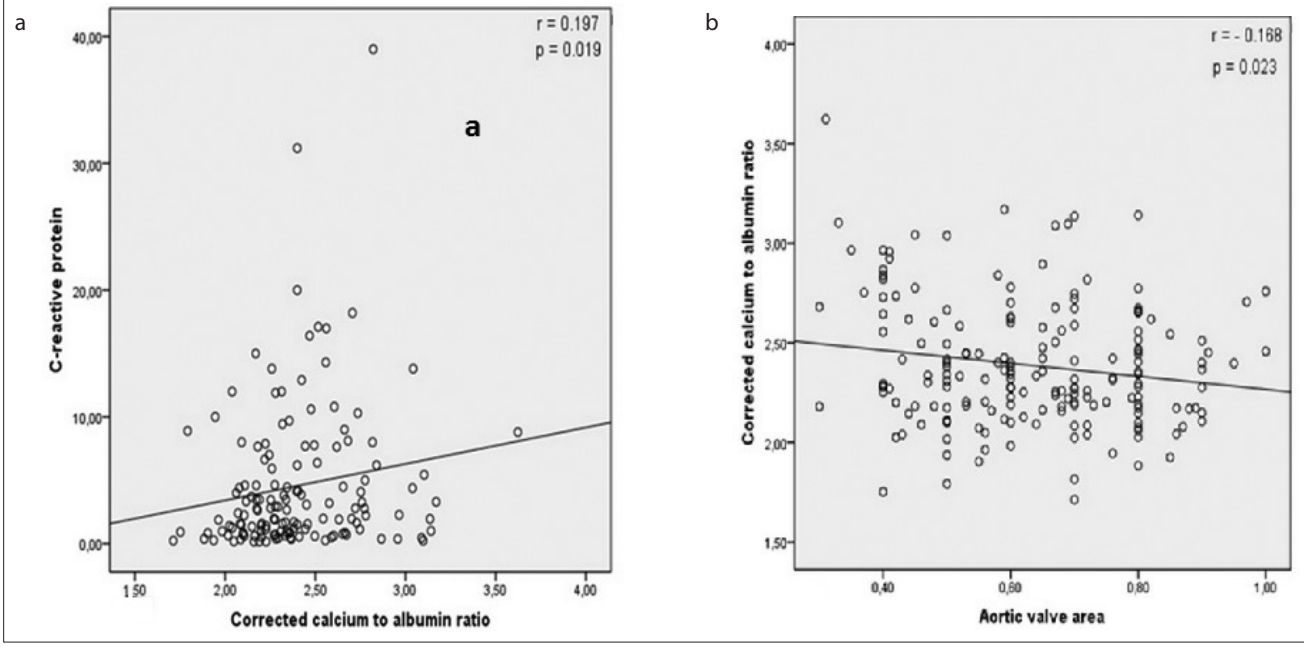


Table 2. Comprasion of baseline characteristic of severe and very severe aortic stenosis

Variables	Severe Aortic Stenosis (n=145)	Very Severe Aortic Stenosis (n=40)	p
Age, (years) (mean ±std)	77.68±7.47	78.55±7.78	0.532
Hypertension, n (%)	110 (75.8)	33 (82.5)	0.254
Diabetes mellitus, n (%)	13 (32.5)	46 (145)	0.534
Creatinine, mg/dl, (median–IQR)	0.93 (0.39–1.44)	0.89 (0.4–1.5)	0,351
LDL–C, mg/dl, (median–IQR)	98.58 (36.2–358)	103.10 (31.1–165)	0.644
HDL–C, mg/dL, (median–IQR)	46 (12–103)	47.5 (16–76)	0.765
Triglyceride, mg/dL, mean±std	102 (30–511)	99.5 (34–295)	0.999
CRP, (median–IQR)	4.3 (0.17–20)	5.79 (0.24–30)	0.039
Calcium (mg/dL) (median–IQR)	9 (7–12.17)	8.91 (6.91–11.36)	0.182
cCalcium (mg/dL) (median–IQR)	9.2 (7.08–12.17)	9.16 (6.99–11.92)	0.435
Albumin (gr/dL) (mean±std)	3.90±0.34	3.69±0.31	<0.001
Calcium/albumin ratio (mean±std)	2.33±0.25	2.36±0.33	0.548
Corrected calcium/albumin ratio (mean±std)	2.37±0.28	2.43±0.40	0.341
LVEF, %, (mean±std)	51.35±15.38	59.30±6.41	0.002
Maximum gradient, mmHg	77 (36–105)	132.5 (87–187)	<0.001
Mean gradient, mmHg	45 (20–64)	72 (58–114)	<0.001
Aortic valve area (cm^2), (median–IQR)	0.66±0.15	0.47±0.10	<0.001
Logistic EuroSCORE (%)	21.57±15.43	21.77±13.61	0.944
Society of Thoracic Surgeons score (%)	7.12±4.73	6.24±3.38	0.271

CAD: coronary artery disease; cCalcium: corrected calcium; CRP: C–reactive protein; HDL: high–density lipoprotein; LDL: low–density lipoprotein; LVEF: left ventricular ejection fraction; WBC: white blood cell

in patients with degenerative AS, such as atherosclerotic heart disease (19). A later study revealed that CRP levels measured at intervals in patients with asymptomatic AS playeda prognostic role in the severity, progression, and clinical outcomes of the disease. In the same study, it was also reported that long-term survival rates are lower in AS characterized byhigh CRP levels (20). Another important marker of chronic inflammation is albumin, which use has been recommended in clinical practice in recent years as it shows the fragility of AS patients (9, 10). Serum albumin, a negative acute-phase protein, is insufficiently produced by the liver during inflammation (13). Similarly, several other studies established that low albumin levels were related to an increased risk of cardiovascular mortality and morbidity (21). In their study that includedpatients who underwent TAVI for AS, Yamamoto et al. (22) detected higher all-cause and cardiovascular mortality rates after the procedure and during their 1-year follow-up in individuals whose pre-procedure albumin value was <3.5 mg/dL. Another striking point in this study is that the log EuroScore and the The Society of Thoracic Surgery Risk Score STS were considerably higher in the group with low albumin levels. However, different from our study, the peak velocity and the mean aortic gradient werefound to be higher in the group with high albumin levels, and the valvular area was observed to be comparable between both groups (22). In a study by Koifman et al. (23), while mean aortic gradients were found to be comparable in individuals with a low serum albumin level, AVA was found to be smaller in the group with a low albumin level. In their study, Bogdan et al. (9) have observed comparable mean and maximal aortic gradients and AVAs between the groups with low and high albumin levels. In our study, while the albumin level was negatively correlated with the mean and maximal aortic pressure, a positive correlation was observed with AVA. Recently, the Valve Academic Research Consortium committee hassuggested adding the"fragility"status of patients to classical pre-operative risk factors in patients treated for AS (10, 24). Aserum albumin level <3.5 g/dL has been accepted as a fragility indicator according to these criteria (10, 24).

Additionally, albumin is the most important transporter of calcium in the blood. Forty percent of calcium is transported as albuminbound. Therefore, for an exact assessment of the total calcium level in the blood, albumin levels should be also known

Table 3. Logistic regression analysis of predictive factors for AS

Variables	Odds Ratio (OR)	95% Confidence Interval for OR	p
Calcium (mg/dL)	1.315	0.833–2.075	0.240
cCalcium (mg/dL)	1.321	27.065–219.1	0.224
Albumin (gr/dL)	2.263	1.180–4.233	0.016
Corrected calcium/ albumin ratio	0.010	0.002–0.049	0.001
Calcium/albumin ratio	0.011	0.003–0.051	0.001
CRP (mg/dL)	0.991	0.899–1.093	0.042
LDL (mg/dL)	1.013	1.005–1.021	0.022

Logistic regresionanalysis of predictive factors for very severe aortic valve stenosis
in patients with severe aortic stenosis

Variables	Odds Ratio (OR)	95% Confidence Interval for OR	p
Calcium(mg/dL)	1.121	0.705–1.782	0.630
cCalcium (mg/dL)	1.189	0.749–1.887	0.462
Albumin (gr/dL)	6.134	1.967–19.136	0.001
Corrected calcium/ albumin ratio	4.613	0.930–22.876	0.047
Calcium/albumin ratio	10.342	1.252–24.296	0.030
CRP (mg/dL)	1.025	0.951–1.105	0.514
LDL (mg/dL)	0.999	0.989–1.008	0.817

*Values set in bold indicate p<0.05

(14). Calcific vascular and valvular heart diseases are known to have several characteristics in common with bone structure remodeling. Therefore, studies have been conducted showing the relationship between AS and theparathyroid hormone (PTH) and Vitamin D levels, which are the main actors of serum calcium, and the phosphorus levels and bone metabolism, which are the main components of the bone structure. In their study, Akat et al. (7) found higher calcium levels in patients withAS without CAD and with normal renal functions than in healthy individuals. Also, in the same study, it was observed that the ratio of the calcium level and the Vitamin D level to PTH washigher in patients with AS. In their study, again in individuals with normal renal functions, Linhartová et al. (25) detected higher serum PTH levels and lower Vitamin D levels in patients with AS. In the same study, while serum calcium levels showed a trend toward being higher, thisdid not reach statistical significance. At the end of this study, the authorsconcluded that the disregulation in the calcium and phosphate metabolism may be effective in the pathogenesis of AS (p=0.06). In AS patients, Yang et al. (26) found higher calcium, phosphate, alkaline phosphatase, PTH, and osteocalcin levels, which are important markers of the bone structure mineral turnover in the blood. Furthermore, a study in patients who used a regular calcium treatment did not detect progression in the aortic valve calcification on computerized tomographic assessment during a 4-year follow-up (27).

Albumin is quantitatively the most important plasma protein, and the synthesis and serum concentrations are regulated by a variety of factors. The decrease inalbumin levels reflects a variety of conditions, including malnutrition, systemic inflammation, heart failure, and hepatic and renal pathologies, and it is known that among patients with chronic diseases, including heart disease, lower serum albumin levels correlate with poor outcome (28, 29). Given that AS is also a chronic disease and, as mentionedabove, is an inflammatory process, low albumin levels in patients canbe expected. Considering that albumin is the most important transporter protein of calcium ions, assessing the albumin-corrected calcium levels during the blood calcium level measurement is important as it shows true calcium levels in this patient population. For the purposes of associating with chronic disease states, indicating albumin-corrected calcium levels may be more reasonable. It has been demonstrated that elevated calcium levels and the higher CAR emerged as novel parameters andwere strongly associated with all-cause mortality in patients with stable CAD. It can be assumed that the results of this study support our findings. However, more data are required to confirm the association between the all-cause mortality and the CAR in patients with AS (30). In daily practice, however, its measuring using an equation may be forgotten. In these patients, a simple CAR may be more important as it shows both the level of AS and inflammation state.

Our study has a few limitations. First, it is a single-center study with a retrospective design. The fact that Vitamin D and PTH levels, which are known to have an effect on calcium metabolism, were not studied in patient groups is an important drawback. Another important limitation is the low number of patients included into the study and the lack of patients with mild and moderate AS. Also, the fact that the serum calcium phosphate levels and phosphate levels were not studied may be considered as a drawback. Furthermore, the fact that only one serum level was studied in patients, that the mean of work-ups within the year was not used, and that other inflammation markers (interleukins, fibrinogen, etc.) were not studied may also be considered as limitation.

CONCLUSION

The present study detected an important relationship between the CAR and the severity of AS. We also detected itscorrelation with CRP and that it wasan important inflammation marker. Consequently, the CARthat emerged as a novel parameter can be calculated using simple biochemistry tests in daily practice andcould benefit other techniques for determining the severity of AS. Larger studies on this subject are needed.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Yıldırım Beyazıt University (Date: 05.03.2018, No: 56).

Informed Consent: Verbal informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - S.S., Y.A.; Design - Y.A.; Supervision - E.B.; Data Collection and/or Processing - S.S., S.B.; Analysis and/or Interpretation - Y.A., H.A.; Literature Search - Y.A., S.S.; Writing Manuscript-Y.A.; Critical Review - E.B.

Conflict of Interest: The authors have no conflicts of interest to declare.

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Original Research

Protective Effect of Proanthocyanidin against Methotrexate-Induced Testicle Damage in Rats

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ABSTRACT

Objective: Methotrexate (MTX) isawidely used chemotherapeutic agent showing side effects such as hepatotoxicity and also testicular damage due to an increase in the production of reactiveoxygen species. Proanthocyanidins (PACs) are known as natural antioxidants that have protective effects against oxidative stress. This study investigates the protective effect of PAC against the MTX-induced testicular damage.

Methods: Twenty-six male Wistar albinorats were divided into fourgroups. Saline (2 cc/kg) was administered to the control group, and PAC (200 mg/kg) was administered to the PACgroup for 7 days. The MTX group received a single dose of MTX (20 mg/kg) on Day 3. A total of 200 mg/kg of PACfor 6 days was administered to the PAC+MTX group, and the group also received a single dose of 20 mg/kg of MTX on Day 3. The animals were sacrificed on Day 8. Analyses were performed, in addition tolight and electron microscopic examinations on the testicular samples.

Results: Histopathological findings such as thinning of the seminiferous tubule epithelium and decrease in spermatogonial cells were detected in the MTX group. Electron microscopic findings indicated similar damage. The MDA levels increased in testicular tissues, while the SOD and GPX levels decreased significantly. The tissue damage and deterioration in biochemical parameters were decreased in the PAC+MTXgroup.

Conclusion: The results show that PAC does not completely prevent damage to the testicles caused by MTX but that it has an important protective effect.

Keywords: Methotrexate, proanthocyanidin, testis

INTRODUCTION

Methotrexate (MTX), a folic acid precursor, is widely used in the treatment of malignant tumors and some non-neoplastic diseases. Chemotherapeutic drugs such as MTX act on the S phase of the cell cycle by inhibiting the synthesis of DNA precursors. As a result, organs with high mitochondrial activity, such as the testicular tissue, become targets of these drugs (1). It has been reported that the drugs used in cancer treatment cause a decrease in the diameter of the lumen of the seminiferous tubule, and the degeneration and shedding of germ cells (2). Oxidative stress plays an important role in testicular damage and pathogenesis resulting from the use of MTX (2, 3).

Oxygen-derived free radicals or reactive oxygen species (ROS) are produced during normal metabolism and energy production of the body. However, if ROSs exceed the antioxidant capacity of the biological system, they cause an imbalance, known as oxidative stress, due to metabolic and other environmental factors. This can damage biological molecules, such as lipids, proteins, polysaccharides, and DNA. Scientific interest in antioxidant compounds, especially those from plants, has been increasing over recent years. The discovery of the protective function of phytonutrients against cancer and other neurodegenerative diseases, and the growing concern about synthetic antioxidants, have made natural antioxidants more attractive (4).

Proanthocyanidins (PACs) are natural antioxidants found in the seeds, flowers and shells of fruits, vegetables, nuts, and especially grapes (*Vitisvinifera*) (5). PACshave biological and pharmacological properties and therapeutic effects against free oxygen radicals and oxidative stress (6, 7). Abundant amounts of grape seed extract containing PAChave been shown to protect against cardiovascular diseases, nephropathy, atherosclerosis, and neuropathy (8-12). In addition, its anti-apoptotic, anti-inflammatory and antimicrobial effects have been demonstrated through animal culture studies, as well as its anticarcinogenic effects (13) through cell culture studies (9-11, 14).

In this study, the effects of PAC on the testicles of rats who were administered MTX were investigated through light microscopy, electron microscopy, and on a biochemical level.

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Table 1. Experiment schedule

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control, n=5	Sa	Sa	Sa	Sa	Sa	Sa	Sa
PAC, n=7	PAC	PAC	PAC	PAC	PAC	PAC	PAC
MTX, n=7	Sa	Sa	MTX+Sa	Sa	Sa	Sa	Sa
PAC+MTX, n=7	PAC	PAC	MTX+PAC	PAC	PAC	PAC	PAC

PAC: proanthocyanidin, 200 mg/kg (gavage); MTX: methotrexate, 20 mg/kg (intraperitoneal); Sa: saline, 2 cc/kg (gavage)

METHODS

In our study, 26 young adult Wistar albino rats with a mean age of 5-6 months old were used. The animals were housed in wire cages in rooms where the ambient light was set to 12-hour darkness and 12-hour light, and the ambient temperature was fixed at 21±1°C. During the experiment, all animals were fed ad libitum. All animals were weighed, and the weights were recorded at the beginning of the study and before the sacrifice. All the procedures carried out in our study were approved by Animal Experiments Local Ethics Committee of Kahramanmaraş Sütçü İmam University (decision date 05.05.2015, decision number 07).

All chemicals used in our study were of the analytical grade and were from the Sigma Chemical Company (SigmaAldrich; St. Louis, Missouri, USA).

Experimental Groups

The experimental animals were divided into fourgroups: thecontrol group, PACgroup, MTXgroup, and MTX+PAC group, where the control group had 5, and other groups had seven rats each (Table 1).

Collecting the Tissues

All animals were sacrificed 24 hours after the last administration (day 8). The animals' testicles were removed, cleaned from the surrounding tissues, and weighed. For each animal, the right testicles were placed in 10% neutral formaldehyde for a follow-up with light microscopy. A small fraction of the left testicles was placed in a 5% glutaraldehyde solution for electron microscopy, and the remaining part was stored at −20°C for biochemical procedures.

Calculation of Testicle Weight Index

The testicle weight index (TWI) values for each animal were determined by calculating the body weight measured on the last day of the experiment and the sum of the right and left testicle weights of the same animal according to the following formula.

TWI= [(right+left testicle total weight)/body weight] × 100

Tissue Biochemical Analysis

The tissues stored in a cold environment (−20°C) were brought up to +4°C just before the experiment. The dissolving tissue samples were weighed in their frozen form and put in glass tubes. Superoxide dismutase (SOD), glutathione peroxidase (GPX), and malondialdehyde (MDA) analyses were performed on the tissues.

Determination of MDA

The MDAof the homogenates was determined spectrophotometrically by measuring the existence of thiobarbituric acid reactive substances (TBARS), as defined by Altintas et al. (15). Phosphoric acid (1%) at 3 mL and 0.6% thiobarbituric acid solutionat 1 mlwere added to 0.5 ml of homogenate. The mixture was heated in boiling water for 45 min. After the mixture was cooled, the colored part was extracted into 4 mL of n-butanol. The absorbance was measured by a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 nm. The amount of lipid peroxides was calculated as the TBARS of lipid peroxidation.

Determination of the SOD Activity

Total SOD activity was determined as defined by Parlaktas et al. (16). Theprinciple of this method is the prevention ofnitrobluetetrazolium (NBT) reduction by the xanthine-xanthineoxidase system as asuperoxide generator. One unit of SOD was defined as the amount of enzyme to cause 50% inhibition in the NBT reduction rate. The results were given in Units per gram of protein.

Determination of the GPX Activity

The GPX activity was measured as defined by Parlaktas et al. (16). An enzymatic reaction in a tube containing nicotinamideadenine dinucleotide phosphate (NADPH), glutathione (GSH), sodium azide, and glutathione reductase was initiated by adding H_2O_2; the change in absorbance at 340 nm was observed using a spectrophotometer. The results were given in Units per milligram of protein.

Preparation of Tissues for Light Microscopy

After the 10% formaldehyde fixation, paraffin blocks were prepared by routine procedures on a tissue-monitoring device (TP 1010; Leica, Wetzlar, Germany).

Sections that were 5-6 µ thick were taken from the prepared paraffin blocks with a microtome (RM2245; Leica, Wetzlar, Germany) device. The preparations were stained with hematoxylin-eosin (HE), analyzed under a light microscope, and photographed. For assessing spermatogenesis in testicular tissues, the Johnsen score has determined. A minimum of 50 tubules were evaluated, and each tubule was given a score from 1 to 10.

Measurement of Tubular Diameter (TD) and Tubular Epithelial Thickness (TET)

Light microscopic sections were examined with the microimage software (cellSens Analysis Systems; Olympus, Tokyo, Japan) and

a light microscope (DM 750; Leica, Wetzlar, Germany). In each group, morphometric analyses were performed by measuring the diameters of ten seminiferous tubules of each animal, two measurements per tubule, and through germinal epithelial thickness measurement from four different regions of the same tubule with 10X magnification and averaging them.

Preparation of Tissues for Electron Microscopy

Tissue samples were fixed with 5% glutaraldehyde and 1% osmium tetraoxide solutions prepared with Millonig's phosphate buffer. After dehydration with the alcohol series, the tissues were immersed in the rotator overnight in the embedding material (resin), and the Epon blocks were prepared the next day. The thin sections obtained from the blocks were placed on a copper grid and after being contrasted with uranyl acetate and lead citrate, they were analyzed with Transmission Electron Microscopy (JEM 1400; Jeol, Massachusetts, USA), and photographs were taken.

Statistical Analysis

Biochemical data were analyzed using the Statistical Package for the Social Sciences 22.0 package program (SPSS IBM Corp.; Armonk, NY, USA). An analysis of variance (ANOVA) test was used to assess whether there was a significant difference between the groups' averages. Values with a p-value <0.05 were considered statistically significant. Post-hoc tests were performed to assess the difference between the groups in the parameters that resulted in a significant difference between the group averages. Taking the homogeneity of the variances of the groups into account, the Tukey test was applied for the parameters of SOD and GPX, which had a greater value than p=0.05. For the MDA with a p-value <0.05, the Games Howell test was applied to see among which groups the group averages differ significantly.

The statistical SPSS 22.0 package program was used forthestatistical evaluation of tubal diameter and epithelium thickness measurements. The normal distribution of the numerical data in this parameter was tested with the Shapiro-Wilk test. The ANOVA test and the Least Significant Difference (LSD) test were used for comparison of the variables that fit normal distribution.

RESULTS

Testis Weight Index

The difference between the TWI values of the groups was not statistically significant (p=0.447; Table 2).

Biochemical Findings

The levels of SOD, GPX, and MDA in the testicular tissues of the groups are shown in Table 3.

While SOD significantly decreased (p<0.001) in the MTX group compared with the control group, it increased (p=0.002) again in the PAC+MTX group, and its difference from the control group became insignificant (p=0.096). There was also a significant difference between the MTX group and the PAC group (p<0.001).

The results of GPX were foundsimilar to SOD. The level of GPX in the PAC+MTX group was high (p=0.002), while in the MTX group, there was a decrease in the GPX level compared to both the control group (p=0.015) and the PAC group (p=0.003). The difference between the PAC group and the PAC+MTX group was not significant (p=0.952).

Table 2. Testicle weight index according to groups of rats (%)

Group	Mean±SD
Control	1.2841±0.1304
PAC	1.2232±0.1090
MTX	1.3122±0.1071
PAC+MTX	1.2182±0.1354

PAC: Proanthocyanidin; MTX: Methotrexate

Table 3. Tissue biochemical values of the experimental groups (mean±standard deviation)

Group	SOD (IU/mg)	GPX (IU/mg)	MDA (nmol/mg)
Control	10.9076±1.3813	0.0555±0.0117	1.5715±0.3216
PAC	12.6453±1.8946	0.0506±0.0027	1.9864±0.5953
MTX	4.5552±1.0220	0.0289±0.0078	4.0179±0.4265
PAC+ MTX	8.4570±2.0908	0.0497±0.0039	2.7004±0.4797

PAC: Proanthocyanidin; MTX: Methotrexate

There was no significant MDA difference between the control and PAC groups (p=0.468), but it was significantly higher in the MTX group than the control group (p<0.001). In the PAC+MTX group, it decreased with respect to the MTX group (p<0.001). The difference between the PAC group and the PAC+MTX group was not significant (p=0.050). The increase in the PAC+MTX group was significant compared to the control group (p=0.003).

Light Microscopic Findings

Histological analysis

Samples from the control group and the PAC group showed a normal testicular histology (Figure 1). Thinning and vacuolization in the seminiferous tubule, degeneration in the spermatogonium cells and shrinkage of the nuclei, and interstitial edema and congestion in some vessels were observed in MTX group (Figure 2). In MTX+PAC group, there was reduced tubular damage, the spermatogenic cells were preserved and the spermatogenesis continued, while the change in the spermatogonia was not as severe as in the MTX group, although some tubular damage in several tubules persisted. In the interstitial area, although there was a small amount of edema, no congestion was seen (Figure 3).

Johnsen score analysis

According to the score results, in the PAC group, there was no difference according to the control group, statistically (p=0.116). The MTX group showed impaired spermatogenesis, and this difference was statistically significant (p<0.001). The MTX+PAC

Table 4. Johnsen scores of the experimental groups

Group	Johnsen Score (Mean±SD)	p
Control	9.529±0.13	
PAC	9.210±1.27	0.116
MTX	6.975±0.475**	<0.001
PAC+MTX	8.903±0.722	0.903

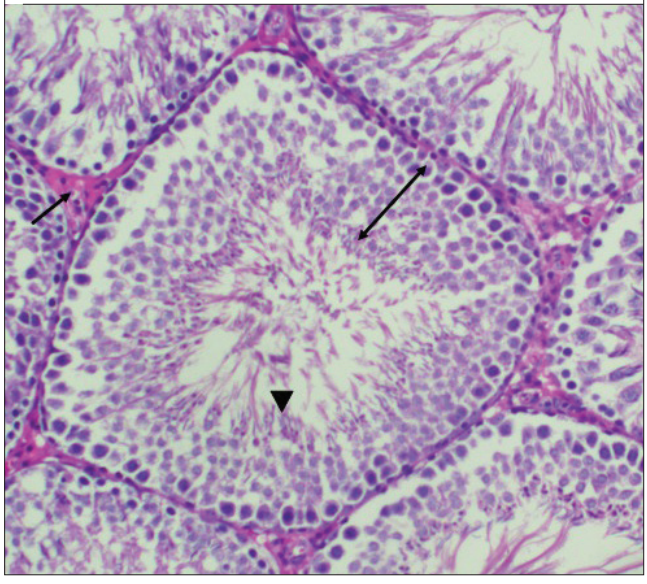
**: p<0.05 compared to control group by ANOVA test
PAC: Proanthocyanidin; MTX: Methotrexate

Table 5. Tubular diameter (TD) and tubular epithelial thickness (TET) measurements

Group	TD (Mean±SD)	TET (Mean±SD)
Control	317.0134±26.4338	38.6471±3.9864
PAC	309.8520±26.8232	35.3019±5.3264
MTX	236.6670±18.4465	25.2050±4.3498
PAC+MTX	334.3897±23.6394	32.9007±2.6315

PAC: Proanthocyanidin; MTX: Methotrexate

Figure 1. The control group light microscopic image revealed normal seminiferous tubules and spermatogenic cell lines of a normal structure (double-headed arrow) on its wall, a normal interstitial area (arrow), and normal spermatids (arrowhead). H&E



group was similar to the control and PAC groups, andthere was no difference statistically (p=0.093) (Table 4).

Tubule diameter and epithelium thickness measurements

While the diameter of the seminiferous tubules was significantly lower in the MTX group than in the control group (p=0.001), the PAC+MTX group maintained values close to the control group. Testicular epithelium thickness was again thinner in the MTX group than in the control group (p=0.001) and was normal again in the PAC+MTX group (Table 5).

Figure 2. The methotrexategroup light microscopic image revealed edema in the interstitial area (arrowhead), thinning in the seminiferous tubule wall (double-headed arrow), and occasional vacuolization (thin arrows) in the seminiferous tubule wall. H&E

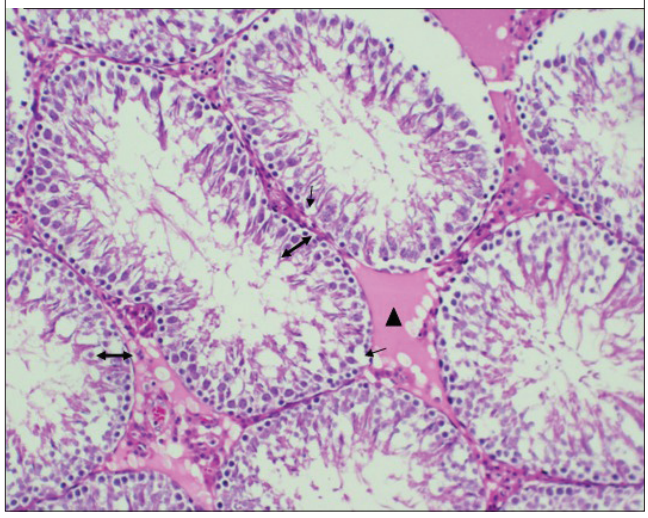
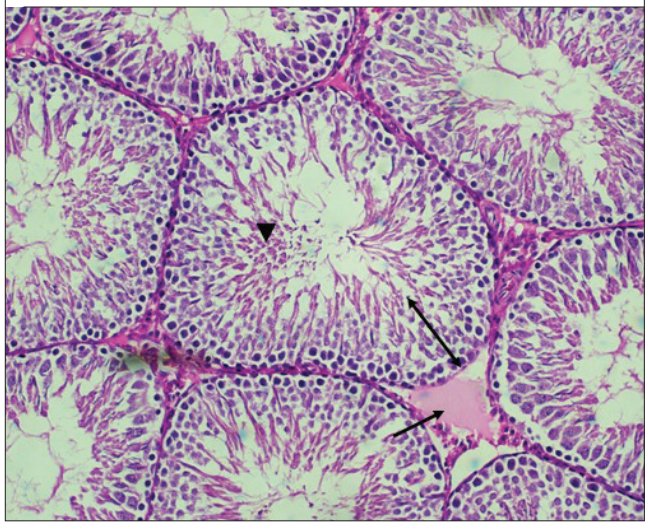


Figure 3. Methotrexate+proanthocyanidin group light microscopic image showed almost normal seminiferous tubule structures (double-headed arrow), preserved spermatogenic cells, continuing spermatogenesis and spermatozoon (arrowhead) in the tubule lumen, and a mild edema in the interstitial area (arrow). H&E



Electron Microscopic Findings

In the control group, normal testicular structures were observed in general (Figures 4, 5). In MTX group, the membranepropria surrounding the seminiferous tubule showed thickening, an increased amount of collagen fibers, and irregularity in the basal lamina (Figure 6). There were indentations between the spermatogonium cells in the tubule epithelium and degradation of the Sertoli cell nucleus (Figure 7). It was observed that the agranular endoplasmic reticulum cisternae in the Sertoli cells

Figure 4. Control group: Sertoli cells (SC) and Sertoli nuclei (SN), spermatogonium (Sg), and primary spermatocytes (PSt) were seen on the membrana propria (MP) surrounding the seminiferous tubule, on the myoid cells (My), and the seminiferous epithelium on the basal lamina (arrowhead). Nucleus (N), mitochondria (m).

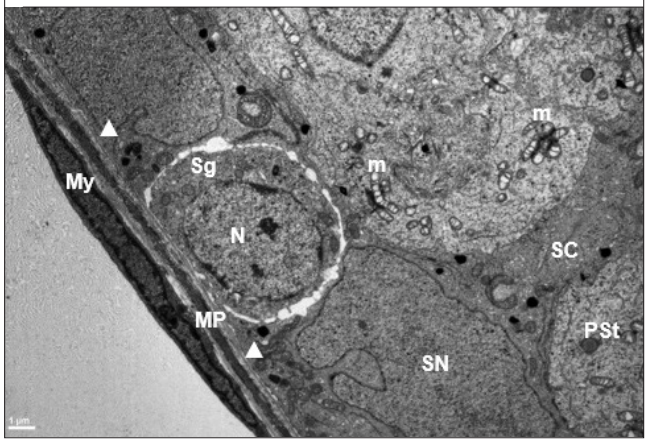


Figure 5. Control group: Normal looking spermatocytes (St) and nuclei (N) are seen located near the seminiferous tubule lumen. The acrosomal vesicle formation (arrows) and tail formation (arrowhead) developing around the nucleus were noteworthy.

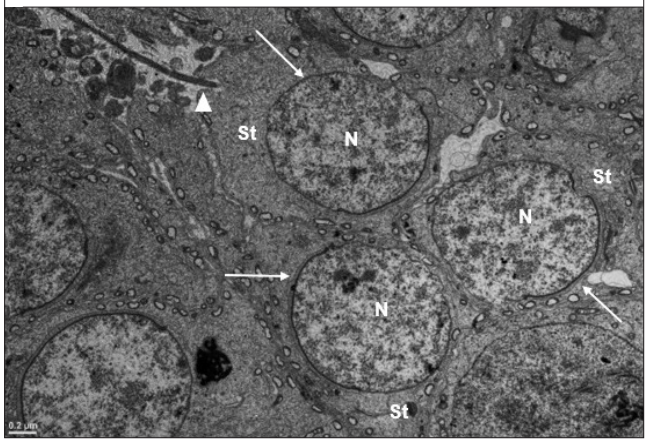


Figure 6. Methotrexategroup: Thickened membrane propria (MP) on the degraded basal lamina and a degenerated myoid cell (My) are observed. Occasional large lipid droplets (L) are seen.

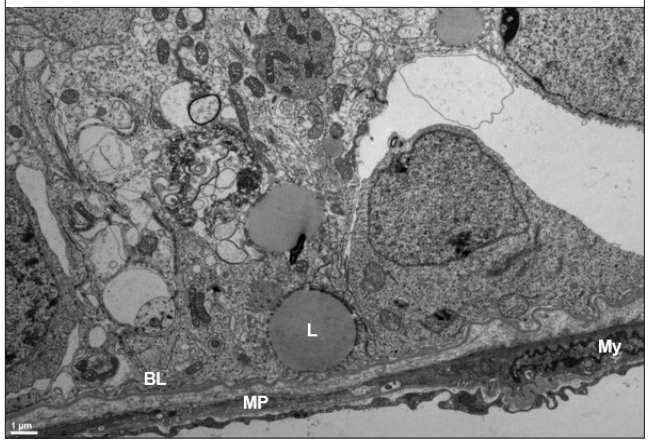
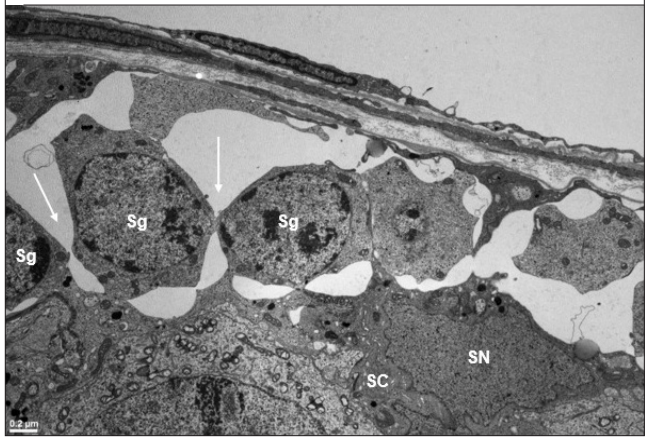


Figure 7. Methotrexategroup: Indentations (arrow) among the spermatogonium (Sg) cells in the seminiferous tubule epithelium were observed, as well as corruptions in the morphology of the Sertoli cell (SC) and the nucleus (SN).



expanded, while lysosomes and lipid droplets increased. Corruptions were observed in the structures of primitive spermatocytes in the seminiferous tubule epithelium, and apoptotic cell structures were seen in the mitochondria, along with some shrinking (Figure 8). Fragmented Leydig cells and nuclei in the interstitial area and small lipid droplets were worthy of note (Figure 9). The PAC group images were similar to those in the control group (Figure 10).When MTX and PAC were administered together in the MTX+PAC group, the pathological findings in the MTX group partially disappeared (Figure 11).

DISCUSSION

Medicines used in chemotherapy generally have adverse effects on all cells in division (17). MTX, a folic acid antagonist, is used in the treatment of diseases, such as lymphoma, osteosarcoma, neck tumors, lung and breast cancers, and testicular cancer (1, 18). Despite its therapeutic effects, MTX is known to cause thinning of the testicles in the walls of the seminiferous tubules, permanent azoospermia, damage to the sperm DNA, abnormalities in the sperm head morphology, and infertility (19-22).

Seminiferous tubule atrophy in the MTX treatment and apoptosis in spermatocytes are thought to be related to the increase of reactive oxygen radicals, and a number of studies have been conducted on the use of antioxidant substances to reduce these side effects (17, 19-21). MTX also reduces the NADPH in cells, leading to glutathione depletion, thereby weakening the cell against ROS and increasing oxidative stress (23). MTX can damage intercellular connection complexes and cause cells to spill into the lumen.

In our study, the light and electron microscopy findings for the MTX group were similar to those of previous studies, including

Figure 8. Methotrexategroup: Deteriorations in the primary spermatocyte (PSt) structures in the seminiferous tubule epithelium, and mitochondrial shrinkage (arrow) are seen. The apoptotic cells (A) stand out.

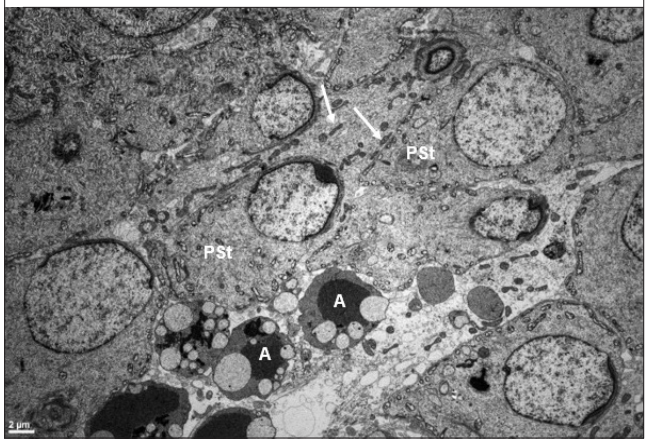


Figure 9. Methotrexategroup: The interstitial area shows lysed Leydig cells (LC), nuclei (LN), and small lipid droplets (arrowhead).

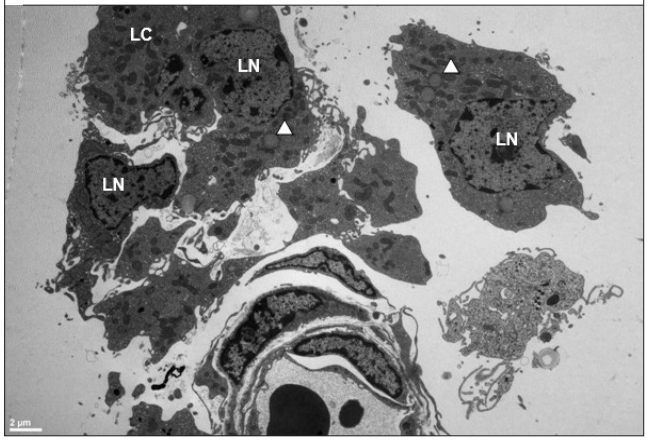


Figure 10. Proanthocyanidingroup:The interstitial area is observed with nearly normal Leydig cells (LC), nuclei (LN), and capillaries (arrow).

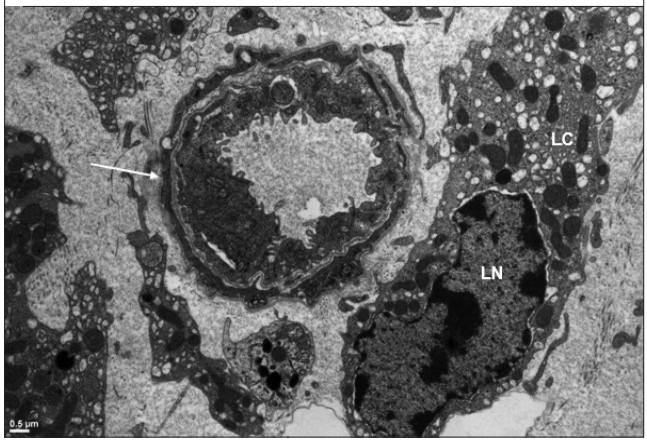
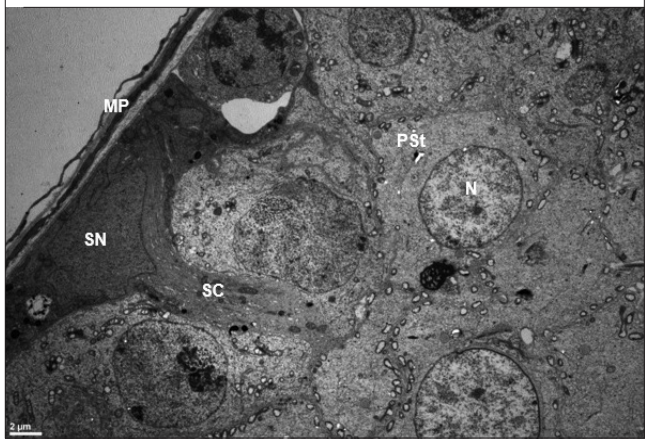


Figure 11. Proanthocyanidin+methotrexategroup. Membrane propria (MP), Sertoli cells (SC) and nuclei (SN), primary spermatocytes (PSt), and nuclei (N) are observed near the seminiferous tubule.



the damage and thinning of the seminiferous epithelium and interstitial edema and congestion (17, 19, 21, 24). Similar to previous studies, the MDA increase in the MTX group and the decreases in the SOD and GPX levels supported these findings.

It is now known that testicular oxidative stress usually causes male infertility in normal or pathological conditions. It has been reported that about 50% of male infertility is due to oxidative stress (25). For this reason, the use of natural antioxidants has become necessary.

In our study, the PAC treatment prevented histopathological findings caused by MTX and improved biochemical parameters that were deteriorated. The electron microscope findings also support these results. There are numerous studies in the literature that show such effects of PAC (5, 12, 14, 25).

Malondialdehyde is a stable end-product of lipid peroxidation generated by ROS, and grape extracts rich in PAC have been shown to reduce elevated MDA levels (9-12, 26).

CONCLUSION

Our study has shown that MTX significantly damages the testicles and that PACsignificantly reduces the severity of such damage, even if it does not completely reverse it.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Kahramanmaraş Sütçü İmam University (decision date 05.05.2015, decision number: 07).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - M.Y., G.Z.; Design -M.Y., G.Z., S.P.; Supervision -M.Y., H.B., N.B.; Resources -H.B., N.B.; Materials -M.Y., G.Z., N.B.; Data Collection and/or Processing -S.P., M.Y., H.B.; Analysis and/or Interpretation -H.B., G.Z., N.B.; Literature Search -M.Y., H.B., S.P.; Writing Manuscript -M.Y., G.Z., H.B., N.B., S.P.; Critical Review -M.Y., G.Z., H.B., N.B., S.P.

Conflict of Interest: The authors have no conflicts of interest to declare.

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Original Research

Association of red Cell Distribution width with Characteristics of Coronary Atherosclerotic Plaques as Detected by Computed Tomography Angiography

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ABSTRACT
Objective: To evaluate the relationship between red blood cell distribution width (RDW) and the severity/morphology of coronary atherosclerotic plaques (CAPs).
Methods: We retrospectively analyzed 572 patients without a history of coronary artery disease (CAD) in whom dual-source 64-slice computed tomography angiography (CTA) was performed due to the suspicion of CAD.
Results: Critical CAPs were detected in 26.9% of subjects. The RDW value was higher in patients with critical CAPs than in those without (13.63±1.28 vs. 14.31±1.58, p<0.001). Patients with any type of CAP regardless of the morphology or severity revealed enhanced RDW levels compared with those with normal coronary arteries (p<0.001). In the multinomial logistic regression analysis, RDW was found as an independent predictor for the presence of severe CAP (odds ratio (OR): 1.40, 95% confidence interval (CI): 1.20–1.63, p<0.001). RDW was also found to be associated with the presence of non-calcified plaque (OR: 1.30, 95% CI: 1.08–1.57, p=0.006) and mixed plaque morphologies (OR: 1.47, 95% CI: 1.19–1.81, p<0.001) after adjusted for other variables.
Conclusion: Our findings suggested that RDW as a simple, available and inexpensive biomarker was significantly associated with both the severity and vulnerable morphology of CAPs in patients undergoing coronary CTA.
Keywords: Coronary atherosclerotic plaque, multidetector computed tomography angiography, red cell distribution width

INTRODUCTION

Red blood cell distribution width (RDW) is a calculation of variableness in the dimensions of circulatory red blood cells. RDW as a costless and routine parameter of standard complete blood count (CBC) test is used for differential diagnosis of anemia (1). Chronic low-grade inflammation and enhanced oxidative stress are associated with defective erythropoiesis and consecutive erythrocyte degradation that results in increased RDW levels (2). It has been previously shown that enhanced RDW levels were related to adverse cardiovascular outcomes in patients with acute coronary syndrome and heart failure (3, 4). Furthermore, increased RDW values were shown to be associated with complex coronary artery lesions during conventional coronary angiography (5).

Multidetector computed tomography angiography (CTA) is an already known non-invasive diagnostic test that provides information about various characteristics of coronary atherosclerotic plaques (CAPs) (e.g., morphology and severity) (6-9). To our knowledge, the association of RDW levels with both the severity and morphological characteristics of CAPs as demonstrated by CTA has not been evaluated yet. The aim of the present study was to evaluate the relationship between RDW and the quantity and quality of CAPs as detected by dual-source 64-slice CTA in patients without a history of CAD.

METHODS

Study Population

A total of 572 patients in whom coronary dual-source 64-slice CTA was performed due to the suspicion of coronary artery disease (CAD) following a detailed clinical and laboratory evaluation were enrolled between January 2009 and June 2011. The mean age of the patients was 55±11 years, and 47.6% were men. Exclusion criteria were history of a documented CAD, history of blood transfusion, hematologic diseases including anemia, renal

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dysfunction (serum creatinine ≥1.5 mg/dL), hepatic disorders, malignancy, acute and/or chronic inflammatory and/or infectious diseases, and surgery or trauma within the previous month. The study was approved by our Institutional Ethics Committee (Hacettepe University, protocol no.: HEK 08/181) in accordance with the ethical issues as outlined in the Declaration of Helsinki. Written informed consent was obtained from each patient before study enrollment.

Definitions and Risk Factor Assessment

Baseline study parameters including age, gender, body mass index, family history of a premature CAD, smoking habits, history of diabetes mellitus, hypertension, hyperlipidemia, and medications were recorded. Hypertension was defined as a systolic blood pressure of ≥140 mmHg and/or a diastolic blood pressure of ≥90 mmHg in at least two measurements or already taking any antihypertensive agent (10). Dyslipidemia was defined as a total cholesterol of >200 mg/dL or triglycerides of >150 mg/dL. Diabetes mellitus was defined as a fasting plasma glucose level of >126 mg/dL or glucose level >200 mg/dL at any measurement or already taking an antidiabetic medication. Smoking was recorded as positive in only current smokers. Global CAD risk was estimated by the Framingham risk equation.

Biochemical and Hematological Measurements

Peripheral venous blood samples were collected after a 12-hour fasting interval. Biochemistry panel was processed using commercially available assay kits (Hitachi P800; Holliston, MA, USA). A CBC analysis including erythrocyte count, hemoglobin (Hb) level, RDW, mean corpuscular volume, and white blood cell count was calculated within 2 h of specimen collection using a Beckman Coulter (High Wycombe, UK) Gen-S automated hematology analyzer.

Coronary CTA

Coronary CTA studies were performed by using a dual-source 64-slice CTA scanner (Somatom Definition; Siemens, Erlangen, Germany). All participants were in sinus rhythm before scanning. Sublingual nitrate (5 mg isosorbide dinitrate (Isordil®; Fako, İstanbul, Turkey)) was administered 2-4 min before the test for coronary vasodilatation. The coronary angiographic scan was obtained following the injection of 80 mL nonionic contrast medium (350 mg I/mL Iomeprol, Iomeron®; Bracco, Milan, Italy) (adjusted for body weight of 1.25 cc/kg) at a flow rate of 6 mL/s followed by 50 mL saline solution with the same injection rate to wash out the contrast material from the right ventricle. Contrast administration was controlled with bolus tracking. The scan parameters were as follows: detector collimation, 32 mm × 0.6 mm; slice acquisition, 64 mm × 0.6 mm; gantry rotation time, 330 ms; temporal resolution, 83 ms; pitch, 0.2-0.47 adapted to the heart rate; tube-current, 390 m as per rotation; and tube potential, 120 kV. Scanning time was approximately 5.7-8.4 s, depending on cardiac dimensions and pitch, in a single breath held in the craniocaudal direction. Prospective electrocardiogram (ECG) tube-current modulation (ECG pulsing) for radiation dose reduction was used for all patients. Retrospective gating technique was used to synchronize data reconstruction with the ECG signal. The reconstructions were made in all cardiac phases at 50 ms intervals at a slice thickness of 0.75 mm and a reconstruction increment of 0.5 mm. The reconstruction interval with the fewest motion artifacts was selected and used for further analysis.

CTA Evaluation

All CTA data were evaluated by a radiology staff unaware of the clinical data of the patients. The intraobserver agreement was 95% for lesion severity and 96% for lesion morphology. CAP was defined as any clearly discernible structure attributable to the coronary artery wall in at least two independent image planes. The non-significant CAP was defined as stenosis causing ≤50% luminal narrowing, and the significant CAP was defined as stenosis causing >50% luminal narrowing. All of the CAPs were included in the analysis. For categorization of the CAPs, the coronary system was divided into 16 separate segments based on a modified American Heart Association classification using original axial images, thin slice, maximal intensity projections, and cross-sectional reconstructions orthogonal to the long axis of each coronary segment (0.75 mm thickness) (11). For each segment, CAPs were categorized as (1) none, (2) calcified, (3) non-calcified, and (4) mixed (Figure 1).

Statistical Analysis

Data analyses were performed using the SPSS software, version 20.0 (SPSS IBM Corp.; Armonk, NY, USA). Data are expressed as mean ± standard deviation or n (%). Kolmogorov-Smirnov test was used to assess the normal distribution of variables. Categorical and continuous variables between the two groups were compared using chi-square test and independent samples t-test, respectively. Kruskal-Wallis test was used to compare differences in

Figure 1. a-d. Contrast-enhanced multidetector computed tomographic image. (a) Normal right coronary artery; (b) significant, non-calcified plaque at the left main coronary artery extending to the ostium of the LAD (arrow); (c) non-significant, mixed plaque at the proximal segment of the LAD; (d) significant, dense calcified plaque at the middle LAD (arrow). LAD, left anterior descending artery

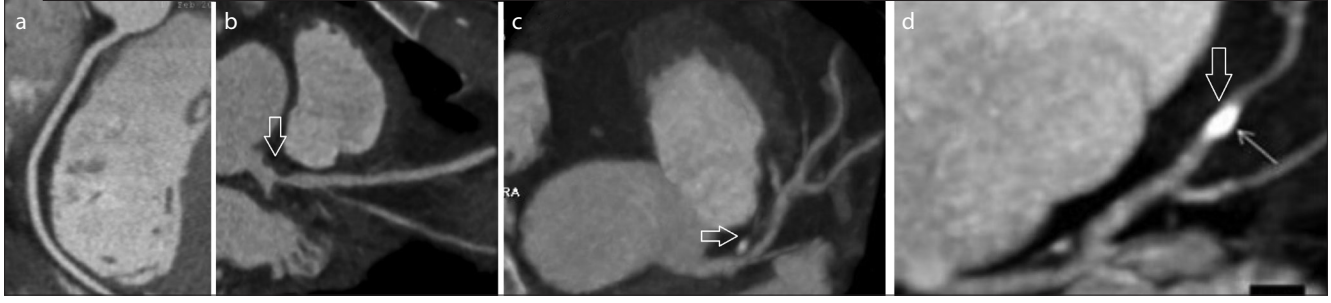


Table 1. Baseline demographic and clinical characteristics of patients due to the severity of coronary atherosclerotic plaques (n=572)

Variables	Non-critical stenosis (n=418)	Critical stenosis (n=154)	p
Age (years)	54±11	58±10	<0.001
Male gender (%)	43.1	59.7	<0.001
BMI (kg/m^2)	27.8±5.0	28.1±4.6	NS
Hypertension (%)	59.3	68.8	0. 038
Diabetes mellitus (%)	13.6	27.3	<0.001
History of smoking (%)	25.4	41.2	<0.001
Family hx of CAD (%)	8.4	11	NS
Total cholesterol (mg/dL)	208±43	202±44	NS
Triglyceride (mg/dL)	145±68	167±88	0.015
HDL-cholesterol (mg/dL)	52±14	50±16	0.023
LDL-cholesterol (mg/dL)	132±38	125±36	NS
Serum creatinine (mg/dL)	0.84±0.19	0.89±0.19	0. 002
Hemoglobin (g/dL)	14.56±1.45	14.31±1.89	NS
RDW (%)	13.63±1.28	14.31±1.58	<0.001
Framingham risk score, n (%)			
Low risk	60.1 (251)	44.4 (68)	
Intermediate risk	29.5 (123)	34.1 (53)	0. 001
High risk	10.5 (44)	21.5 (33)	
Medications			
Aspirin (%)	50	66	0. 001
Statin (%)	34.6	40.5	NS
Beta blocker (%)	22.2	40.5	<0.001
CCB (%)	12.1	17	NS
ACE inhibitor (%)	14.3	20.3	NS
ARB (%)	29.0	32.7	NS

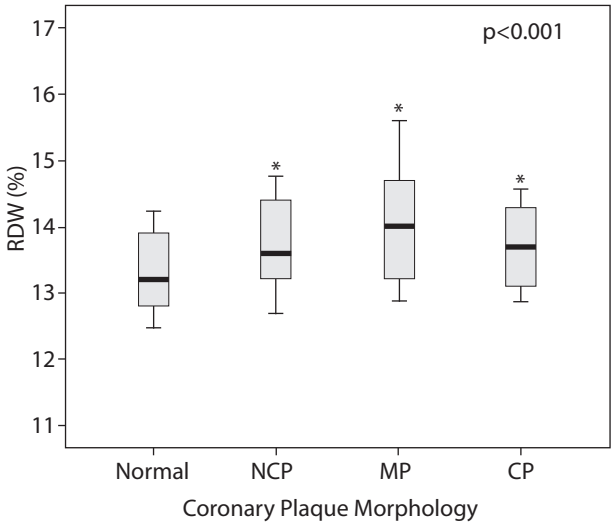
ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker; BMI: body mass index; CAD: coronary artery disease; CCB: calcium channel blocker; HDL: high-density lipoprotein; hx: history; LDL: low-density lipoprotein; NS: non-significant; RDW: red blood cell distribution width

mean values between the groups where there were three or more independent groups. Logistic regression analysis was performed to assess the predictors of the presence and severity of CAPs. A two-tailed p-value <0.05 was accepted as statistically significant.

RESULTS

Significant CAPs (luminal stenosis of >50%) were detected in 154/572 (26.9%) of subjects. The comparison of baseline parameters of the study groups according to the severity of CAPs as detected by CTA is presented in Table 1. Subjects with significant CAP were at an older age and predominantly male. Hypertension, diabetes mellitus, and history of smoking were also more prevalent in subjects with significant luminal stenosis (p<0.05). Moreover, the RDW level was found to be higher in patients with the significant CAP than in those without (13.63±1.28 vs. 14.31±1.58, p<0.001).

Figure 2. Comparison of RDW levels in between patients with normal coronary arteries and different atherosclerotic plaque morphologies



Furthermore, we assessed the relationship between RDW levels and the morphology of the CAPs (Table 2). A total of 345 (60.3%) patients with CAPs in their coronary arteries were stratified due to CAP morphologies as having primarily (defined as >70% of all segments) non-calcified plaque (NCP), mixed plaque (MP), or calcified plaque (CP). Percentages of patients with primarily NCP, MP, and CP were 32.8%, 11.9%, and 15.6%, respectively (Table 2). The RDW level was higher in patients with NCP, MP, and CP than in those with normal coronary arteries (13.96±1.47, 14.37±1.90, 13.80±0.95, and 13.51±1.25, respectively, p<0.001). Furthermore, the RDW level was significantly higher in patients with NCP and MP than in those with CP (13.96±1.47, 14.37±1.90 vs. 13.80±0.95, respectively, p<0.001) (Figure 2).

In the multinomial logistic regression analysis, RDW was found to be an independent predictor for the presence of significant CAPs at coronary CTA (odds ratio (OR): 1.40, 95% confidence interval (CI): 1.20-1.63, p<0.001) (Table 3). In addition to RDW level, age (OR: 1.03, 95% CI: 1.005-1.053, p=0.016), male gender (OR: 1.82, 95% CI: 1.08-3.03, p=0.023), smoking habit (OR: 2.52, 95% CI: 1.52-4.17, p<0.001), diabetes mellitus (OR: 1.83, 95% CI: 1.01-3.32, p=0.047), and serum triglyceride level (OR: 1.003, 95% CI: 1.000-1.007, p=0.049) were also found as significant predictors of the severity of CAP after adjustment for other risk factors (Table 3).

Table 2. Baseline characteristics of patients due to the morphology of atherosclerotic plaques (n=572)

Variables	Normal (n=222)	NCP (n=188)	MP (n=68)	CP (n=89)	p
BMI (kg/m²)	27.3±5.4	28.2±4.2	28.6±4.9	27.9±4.6	0.030
Hypertension (%)	50.9	66.8	62.3	78	<0.001
Diabetes mellitus (%)	9.9	21.6	20.3	24.2	0.002
Smoking (%)	27.5	36	29	22	NS
Family hx of CAD (%)	9.5	10	7.2	7.7	NS
Total cholesterol (mg/dL)	206±40	206±45	204±38	210±52	NS
Triglyceride (mg/dL)	147±66	158±81	156±83	144±71	NS
HDL–cholesterol (mg/dL)	53±14	49±13	52±18	54±15	0.015
LDL–cholesterol (mg/dL)	130±34	130±40	126±32	132±43	NS
Serum creatinine (mg/dL)	0.84±0.19	0.88±0.18	0.82±0.15	0.85±0.24	0.049
Hemoglobin (g/dL)	14.55±1.48	14.49±1.75	14.29±1.64	14.49±1.40	0.04
RDW (%)	13.51±1.50	13.96±1.47	14.37±1.90	13.80±0.95	<0.001
Framingham risk score					
Low risk (%, n)	71.2	37.3	62.9	46.2	<0.001
Intermediate risk (%, n)	21.5	40.5	27.4	38.5	
High risk (%, n)	7.3	22.2	9.7	15.4	
Medications					
Aspirin (%)	43.7	58.5	63.2	65.2	<0.001
Statin (%)	27.6	44.9	33.8	41.1	0.002
Beta blocker (%)	20.3	29.8	29.4	37.1	0.014
CCB (%)	7.7	17	13.2	20.2	0.007
ACE inhibitor (%)	15.3	17.6	14.7	14.6	NS
ARB (%)	24.8	32.4	29.4	38.2	NS

ACE: angiotensin–converting enzyme; ARB: angiotensin receptor blocker; BMI: body mass index; CAD: coronary artery disease; CCB: calcium channel blocker; CP: calcified plaque; HDL: high–density lipoprotein; hx: history; LDL: low–density lipoprotein; MP: mixed plaque (MP); NCP: non–calcified plaque; NS: non–significant; RDW: red blood cell distribution width

In the multinomial logistic regression analysis, RDW was also found to be a significant predictor of NCP (OR: 1.30, 95% CI: 1.08-1.57, p=0.006) and MP (OR: 1.47, 95% CI: 1.20-1.81, p<0.001) after adjustment for other risk factors (Table 4).

DISCUSSION

The major findings of our study included: (1) the RDW level was significantly higher among patients with significant CAPs than among patients with non-significant CAPs, (2) the RDW levels were also higher in patients with predominantly NCP and MP than in those with CP and normal coronary arteries, (3) the RDW level was found as an independent predictor for the presence of significant CAPs, and (4) increased RDW levels were also significantly associated with either non-calcified or mixed morphology of CAPs. To our knowledge, this is the first study evaluating the relationship between RDW and both the severity and morphology of coronary atherosclerotic disease as detected by coronary CTA.

Previously, Lippi et al. (12) reported that proinflammatory biomarkers have been well correlated with the RDW levels independent from age, sex, Hb, and ferritin (12). Recent studies have also confirmed that RDW levels have closely had prognostic value in various cardiovascular diseases (13, 14). Additionally, another study has revealed that RDW might have a diagnostic importance in patients with acute coronary syndrome. This study has demonstrated that RDW has a sensitivity of 79% and a specificity of 50% for diagnosing acute coronary syndrome when the cut-off value is 14% (15). Recently, the association of RDW level with stable CAD has also received attention. Tonelli et al. (16) showed that RDW has been linked to major adverse cardiovascular outcomes in patients with

stable CAD with no concomitant heart failure symptoms. Isik et al. (17) also reported that RDW is significantly associated with the Syntax score as an indicator of the presence and complexity of stable CAD. Some studies have suggested that RDW is associated with cardiac syndrome X, atrial fibrillation, and heart failure (18-20).

The association of RDW with the severity of CAD has not been clarified yet. Among various hypotheses to explain the role of RDW in cardiovascular diseases, inflammation is accused at first (12) since recent findings have suggested that chronic low-grade ongoing inflammation plays a central role in atherogenesis (21). It is previously presented that increased proinflammatory markers, such as C-reactive protein and interleukin 6, have been correlated with the extent of lesions and the severity of CAD (21). Inflammation causes an increment of RDW levels by the suppression of erythropoietin gene expression and proliferation of erythroid progenitor cells, downregulation of erythropoietin receptor expression, or reduction of erythrocyte turnover (22). In addition, proinflammatory molecules block the maturation of erythrocytes, resulting in increased levels of RDW (23).

Table 3. Multinomial regression analysis demonstrating the association between cardiovascular risk factors and critical stenosis as detected in coronary computed tomography angiography

Variables	OR (95% CI)	p
Age, years	1.03 (1.01–1.05)	0.016
Male gender	1.82 (1.08–3.04)	NS
Hypertension	1.02 (0.59–1.76)	NS
Smoking	2.52 (1.52–4.17)	<0.001
Diabetes mellitus	1.83 (1.01–3.32)	0.047
Serum creatinine	1.76 (0.48–6.47)	NS
Triglyceride	1.003 (1.000–1.007)	0.049
HDL–cholesterol	1.01 (0.99–1.03)	NS
LDL–cholesterol	0.99 (0.98–1.00)	NS
RDW	1.40 (1.20–1.63)	<0.001

CI: confidence interval; HDL: high–density lipoprotein; LDL: low–density lipoprotein; NS: non–significant; OR: odds ratio; RDW: red blood cell distribution width

The composition of the CAP is an important predictor of the clinical progression and outcome in CAD. Plaques with abundant lipid and inflammatory cell content are more vulnerable to rupture and are often NCPs with low attenuation in coronary CTA (24). Previous studies showed that NCPs are also associated with the development of ACS more than the other plaque morphologies (25). Russo et al. (26) have demonstrated that among patients with suspected CAD, adverse cardiac outcomes are significantly higher in patients with NCPs and/or MPs than in patients with CPs. In our study, we have found that groups of patients with primarily CPs or normal coronary arteries had lower RDW levels than those of patients who primarily have NCPs or MPs. In addition, the RDW level is found to be an independent predictor for the presence of NCP or MP, but not CP after adjustment for other risk factors.

Our study has some limitations. First, adverse cardiovascular outcomes during follow-up have not been analyzed due to the cross-sectional and retrospective design of the study. Second, this was a single-center study; thus, our findings could not be generalized to whole populations. Third, levels of other parameters that may have effects on RDW including serum levels of iron, vitamin B_{12}, and folic acid have not been simultaneously measured in the present study.

CONCLUSION

Our findings suggested that RDW as a simple, available and inexpensive biomarker was significantly associated with both the severity and vulnerable morphology of CAPs in patients undergoing coronary CTA. These data suggest that RDW might be an easily available biomarker for prediction of both the severity and mor-

Table 4. Multinomial regression analysis demonstrating the association between cardiovascular risk factors and the morphology of atherosclerotic plaque

	Non–calcified plaque		Mixed plaque		Calcified plaque	
Variable	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Age, years	1.07 (1.04–1.09)	<0.001	1.03 (1.01–1.06)	0.016	1.06 (1.04–1.09)	<0.001
Male gender	2.18 (1.33–3.60)	0.002	1.09 (0.57–2.10)	NS	1.59 (0.87–2.89)	NS
LDL–cholesterol	1.001 (0.996–1.007)	NS	0.998 (0.991–1.006)	NS	1.001 (0.994–1.008)	NS
HDL–cholesterol	0.985 (0.969–1.000)	NS	0.993 (0.974–1.013)	NS	1.001 (0.984–1.019)	NS
Hypertension	1.19 (0.74–1.91)	NS	1.10 (0.59–2.05)	NS	2.06 (1.11–3.83)	0.023
Diabetes mellitus	1.45 (0.78–2.70)	NS	1.59 (0.72–3.52)	NS	1.75 (0.86–3.52)	NS
Smoking	1.03 (0.64–1.67)	NS	0.98 (0.51–1.88)	NS	0.61 (0.32–1.16)	NS
RDW	1.30 (1.08–1.57)	0.006	1.47 (1.12–1.81)	<0.001	1.17 (0.93–1.48)	NS

HDL: high–density lipoprotein; LDL: low–density lipoprotein; NS: non–significant; RDW: red blood cell distribution width

phology of coronary atherosclerotic disease in patients undergoing coronary CTA and may prove itself as a component of a scoring system for cardiovascular disease risk stratification.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Hacettepe University (protocol no.: HEK 08/181).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - M.U.Y., D.K., K.M.G.; Design - U.C., H.Y., A.H.A., T.H.; Supervision - H.Y., K.A., N.Ö., U.C.; Data Collection and/or Processing - U.C., M.U.Y., D.K., K.M.G.; Analysis and/or Interpretation - D.K., U.C., H.Y., T.H., K.A.; Literature Search - D.K., U.C., N.Ö., K.A.; Writing Manuscript - D.K., U.C., M.U.Y.; Critical Review - T.H., N.Ö., K.A., H.Y.

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Original Research

Apparent Diffusion Coefficient Values of Renal Parenchyma in Healthy Adults: A 3 Tesla MRI Study

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ABSTRACT

Objective: In this study, we aimed to measure apparent diffusion coefficient (ADC) values of healthy renal parenchyma using diffusion-weighted imaging (DWI) in a 3 Tesla (3T) magnetic resonance imaging (MRI) device.

Methods: Apparent diffusion coefficient values of right and left renal parenchyma were measured in 87 individuals (64 females, 23 males) from DWIs obtained with a 3T MRI device. In the ADC measurement, bilateral renal parenchymal margins were drawn by the free-hand region of interest (ROI) on DWI (b=600 s/mm^2), and ADC_{mean} values were recorded from ROIs on the ADC map.

Results: The ADC_{mean} value of renal parenchyma was 2.21x10^{-3} mm^2/s and 2.2x10^{-3} mm^2/s for the right and left kidney, respectively. Measured ADC values of the right and left renal parenchyma were highly consistent (Intraclass correlation coefficient (ICC=0.968; confidence interval (CI):[0.952–0.979]). ADC values of renal parenchyma were significantly lower in the group of patients older than or equal to 50 years as compared to the group of patients younger than 50 years (p=0.001). There was no significant difference between females and males in terms of the ADC values of renal parenchyma (p=0.161 for the right kidney, p=0.207 for the left kidney). Measured ADC values of the right and left renal parenchyma were highly consistent (ICC=0.968; CI:[0.952–0.979]). There was a strong negative correlation between ADC values of renal parenchyma and age (r=−0.686, p=0.001 for the right kidney; r=−0.759, p=0.001 for the left kidney).

Conclusion: Apparent diffusion coefficient values are quantitative values obtained by DWI, and it is important to understand the ADC values of normal healthy renal parenchyma in order to interpret ADC values in renal pathologies.

Keywords: Apparent diffusion coefficient, diffusion-weighted imaging, kidney

INTRODUCTION

Diffusion-weighted magnetic resonance (MR) imaging is an imaging method based on detecting the random movements of water molecules (1). In the human body, water is in cells and extracellular compartments. While the water molecules in extracellular media show relatively free diffusion, intracellular molecules exhibit relatively limited diffusion. Different tissues of the body have a characteristic cellular structure and proportions of intracellular and extracellular regions, and they also have characteristic diffusion properties. The relative proportion of water distribution between these compartments varies with pathological processes. For example, in intracellular high-grade malignancies, the intracellular ratio increases, and diffusion is relatively restricted. Diffusion-weighted imaging (DWI) provides qualitative and quantitative information about diffusion characteristics (2).

Diffusion-weighted imaging is an advantageous MR imaging method as it does not involve any contrast agents, and it is a non-invasive method, enabling quantitative assessment as well as qualitative assessment. The apparent diffusion coefficient (ADC) is a quantitative value that enables quantitative measurement on the ADC map obtained from DWI. While DWI has previously been performed on the brain for the diagnosis of acute ischemia, it has also become common in abdominal pathologies, and it is now included as an imaging modality in routine MR examinations in many centers. It greatly contributes to diagnosis in pathologies of abdominal organs such as the liver and pancreas (3, 4). Studies have shown that ADC values have been useful in assessing solid kidney lesions, differentiating between benign and malign kidney lesions and the subtype determination of malign solid masses (5-7).

It is important to understand the ADC values of normal healthy renal parenchyma to interpret ADC values in renal pathologies. Although there have been studies conducted with a 1.5 Tesla MRI device on this subject, the number of studies conducted with a 3 Tesla (3T) MRI device is limited in the literature (8). In this study, we aimed to measure the ADC values of healthy renal parenchyma using DWI with a 3T MRI device.

METHODS

Volunteers without abdominal MRI findings who underwent abdominal MR imaging, including DWI for screening purposes due to other reasons, between August 2017 and August 2018 in the Radiology Department of Gaziantep University, School of Medicine, were included in this retrospective study. Written informed consent was provided by all patients prior to the start of

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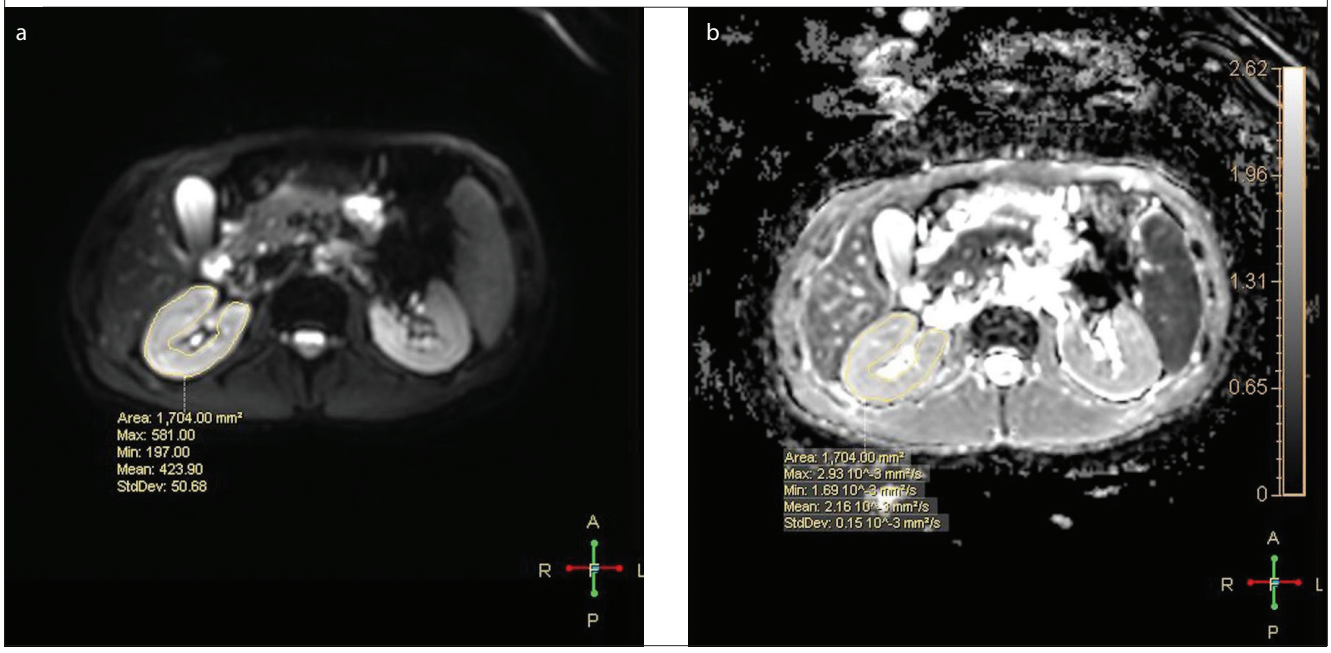
Figure 1. a, b. The measurement of renal parenchyma apparent diffusion coefficient (ADC) values of a 40-year-old male. Axial diffusion-weighted image (a) obtained at a b value of 600 s/mm^2. The ADC map (b) shows that the ADC mean value of the kidney is 2.16x10^{-3} mm^2/s



the study, which was approved by Gaziantep University Ethics Committee (decision no. 2018/246).

Patients with a known kidney disease, hypertension, and diabetes were excluded from the study. The MR examination was performed on a 3T MRI device (Ingenia 3.0 T; Philips Healthcare, Best, The Netherlands), as an abdominal MRI that also includes diffusion MRI. MRI sequence parameters were as follows: axial T2-weighted turbo spin echo with fat suppression, gradient echo in phase and in opposed phase with T1 weighting, diffusion-weighted axial images, and contrast-enhanced dynamic T1-weighted imaging. DWI was acquired with b values of 0 and 600 s/mm^2 and without a contrast agent. Diffusion-weighted sequence parameters were as follows: repetition time/echo time, 1553/61 mS; flip angle, 90°; slice thickness, 5 mm; field of view, 400 x 350 x 270 mm. A diffusion-weighted sequence was obtained at two different b values (b=0 and b=600 s/mm^2) with single-shot echo planar imaging in the axial plane. ADC maps were generated automatically by the device.

The final study population was 87 consecutive patients (64 females, 23 males; age range, 19–80 years; mean age, 47 years). The study population was divided into two groups: younger than 50 years, and older than or equal to 50 years, in order to evaluate the relationship between the ADC values of renal parenchyma and aging. There were 52 individuals younger than 50 years, and 35 individuals older than or equal to 50 years.

In the ADC measurement, bilateral renal parenchymal margins were drawn by free-hand ROI on DWI (b=600 s/ mm^2), and ADC_{mean} values were recorded from ROIs on the ADC map. Measurements were repeated five times for each kidney, and the ADC_{mean} value was calculated and recorded individually for the right and left kidney. Figure 1 shows the measured ADC values of renal parenchyma.

Statistical Analysis

As descriptive statistics, numerical variables were expressed as the mean±standard deviation, and a confidence interval (CI) of 95% was used. Categorical variables were expressed as a number and percentage. Normal distribution of the data was tested by the Shapiro–Wilk test. The Pearson correlation coefficient was used to evaluate the relationship between the ADC values of renal parenchyma and age. The intraclass correlation coefficient was used to compare the values of the right and left renal parenchyma. Gender and ADC relationship was evaluated with the Student's t test. The Student's t test was used for the comparison of ADC values of the group younger than 50 years, and older than or equal to 50 years. The Statistical Package for the Social Sciences for Windows version 22.0 (SPSS IBM Corp.; Armonk, NY, USA) software package was used for statistical analysis, and $p<0.05$ was considered statistically significant.

RESULTS

The ADC_{mean} value of renal parenchyma was 2.21x10^{-3} mm^2/s and 2.2x10^{-3} mm^2/s for the right and left kidney, respectively. Measured ADC values of the right and left renal parenchyma were highly consistent (ICC=0.968, CI:[0.952–0.979]). ADC values of renal parenchyma were significantly lower in the group older than or equal to 50 years in comparison to the group younger than 50 years (p=0.001). There was no significant difference between males and females in terms of ADC values of renal parenchyma (p=0.161 for the right kidney, p=0.207 for the left kidney). There was a strong negative correlation between the ADC values of renal parenchyma and age (r=–0.686, p=0.001 for the right kid-

Figure 2. Strong negative correlation between the apparent diffusion coefficient values of the right kidney parenchyma and age (r=–0.686, p=0.001)

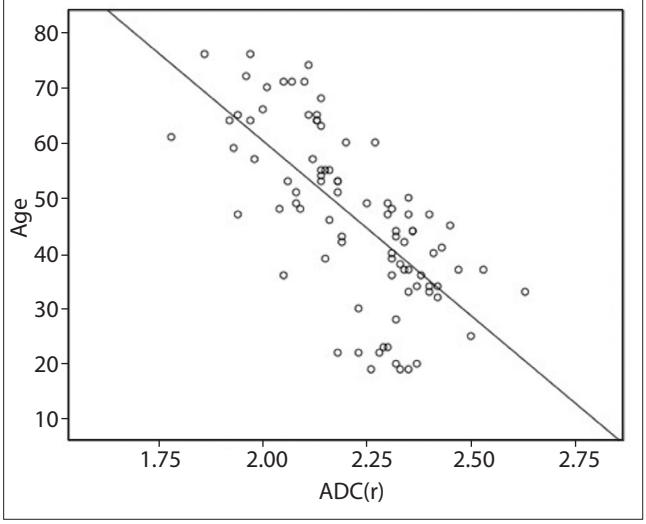
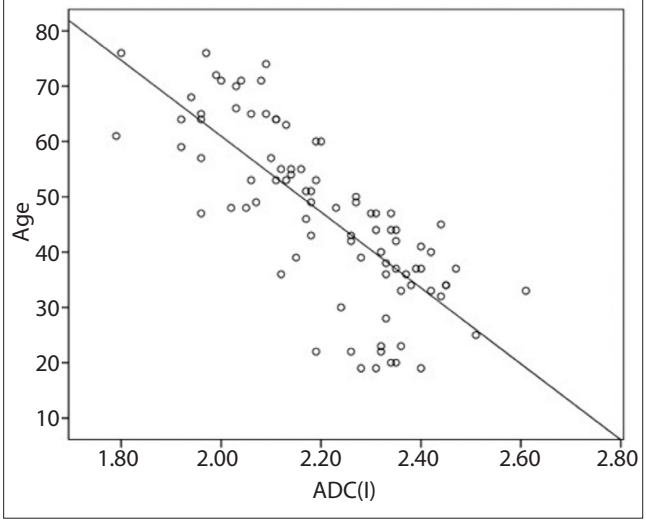


Figure 3. Strong negative correlation between the ADC values of the left kidney parenchyma and age (r=–0.759, p=0.001)



ney; and r=–0.759, p=0.001 for the left kidney). This correlation is shown in Figures 2 and 3.

DISCUSSION

Diffusion-weighted imaging can be used in diagnosing of kidney diseases as of the prenatal period since it is noninvasive and does not require a contrast agent. Including DWI in routine abdominal MRI enables the evaluation of morphological and functional changes together. High ADC values of cystic lesions are useful in differentiating between solid lesions and cystic lesions (9). Necrotic and cystic tumor areas also have significantly lower ADC values as compared to simple cysts (5). In a study conducted by Razek et al. (10), a significant difference was found between the mean ADC values of malignant and benign renal tumors. The authors also reported that using 1.84x10^{-3} mm^2/s cutoff value to predict malignancy had a sensitivity of 89% and specificity of 89%. Diffusion tensor imaging (DTI) is an advanced DWI technique and can be used in many regions of body as other advanced diffusion techniques (11). A study on DTI of the kidney showed that renal fractional anisotropy and renal cortex ADC may be useful to differentiate a diabetic kidney from kidneys of healthy volunteers (12).

Apparent diffusion coefficient values were found to be effective in differentiating between the subtypes of renal cell carcinomas (7, 10). It is possible to distinguish renal oncocytoma from solid renal cell carcinoma RCC using a 1.66 x10^{-3} mm^2/s cutoff value with a 90% sensitivity and 83% specificity (6). In addition, it was also found that DWI was promising in the early diagnosis of renal failure (13).

It can also be a promising method in differentiating diffusion and perfusion problems that develop after renal transplantation (14). It is important to understand the ADC values of healthy renal parenchyma to compare ADC values obtained from DWI in kidney diseases. Therefore, studies have been conducted although the majority of them employed a 1.5 Tesla MRI device. However, the number of studies on this subject employing the 3T MRI device is limited. Hence, it is important to evaluate normal values of renal parenchyma with DWI obtained in 3T MRI devices. DWIs in our study were obtained using a 3T MRI device.

In previous studies, the ADC values of renal parenchyma were reported within a wide range between 1.64 x10^{-3} mm^2/s and 3.54 x10^{-3} mm^2/s, and these values are usually similar to the values found by us as is the case with many of the studies (8, 9, 15-25).

Some of the studies on ADC values of renal parenchyma separated ADC values as the cortex and medulla, whereas others provided a mean ADC value for the entire parenchyma like in our study. Studies that measured ADC values of the entire parenchyma were generally conducted with low b values similar to our study. On the other hand, Yildirim et al. (18, 19) and Murtz et al. (24) also used high b values such as b1000 s /mm^{2}and b1300s /mm^2 while performing DWI and found 1.90x10^{-3} mm^2/s and 1.64x10^{-3} mm^2/s, respectively.

Patients are generally asked to hold their breath while performing DWI to minimize artifacts caused by breathing. In our study, we used respiratory-triggered DWI. The images obtained did not have any artifacts.

Round-shaped ROIs and rectangular ROIs of various sizes were used while conducting ADC measurements in DWI. In our study, free-hand ROI was manually drawn on an axial b600 image, and the ADC_{mean} values seen in the ROI generated on the ADC map were recorded. Therefore, it was intended to include the contribution of the entire parenchyma within the slice to the measurement instead of making measurements in a focal area with small ROIs.

In a study by Suo et al. (26), the mean ADC value of renal parenchyma was found to be 2.23 x10^{-3} mm^2/s, which is very close to the mean ADC value found in our study. It is thought that the similarity between these values can be attributed to the fact that the b val-

ues used in DWI were similar. There was a weak negative correlation between ADC values and age in the study by Suo et al. (26). In our study, there was a strong negative correlation between ADC values and age. It was shown that glomerulosclerosis development was observed, and the renal blood flow decreased with increasing age.

There was no difference between the ADC values of the two kidneys in our study as in other studies. The fact that a difference is not normally expected between the ADC values of the two kidneys can constitute importance in pathologies involving one kidney such that the ADC values of the other kidney can be used as a reference.

It should be noted that the DWI method affects ADC values during their evaluation. In our study, a diffusion-weighted sequence was obtained at two different b values (b=0 and b=600 s/mm^2) with single-shot echo planar imaging in an axial plane. Echo planar imaging is the fastest data collection technique that is also used for dynamic and functional MR imaging. In our study, the scan duration was 2 minutes and 6 seconds.

The b value is the measure of diffusion weight. Although there have been various b values used in other studies, a low b value (600 s/mm^2) was used in our study, which enabled the reduction of motion artifacts and improvement of the signal-to-noise ratio.

This study had some limitations. First, the study was retrospective, and it comprised a small population. Second, the number of males was considerably lower than that of females, and third, measurements were performed by only one radiologist. Measurements were repeated five times, and the mean value of these measurements was used to minimize potential measurement errors.

CONCLUSION

A relationship was not observed between gender and ADC values of renal parenchyma in this study. In addition, there was no significant difference between the right and left kidney. It was found that there was a negative correlation between ADC values and age. Age may also need to be considered in interpreting ADC values in renal pathologies. Studies with larger study populations are required to confirm our findings.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Gaziantep University (decision no. 2018/246).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The author have no conflicts of interest to declare.

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Original Research

The Role of Selvester Score on 12-Lead ECG in Determination of Left Ventricular Systolic Dysfunction Among Patients Receiving Trastuzumab Therapy

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ABSTRACT

Objective: Breast cancer is the most common cancer in women. Trastuzumab is an effective breast cancer agent. The most significant side effect of trastuzumab is left ventricular systolic dysfunction. Selvester score calculated from 12-lead electrocardiography (ECG) has a proven accuracy in predicting left ventricular infarct area and scar volume. We aimed to determine its role in detection of left ventricular systolic dysfunction among trastuzumab-treated breast cancer patients.

Methods: A total of 60 trastuzumab-treated patients were retrospectively included. The patients were grouped into two groups with trastuzumab-induced left ventricular systolic dysfunction (left ventricular ejection fraction (LVEF) <55%) (Group 1) and without (Group 2). The left ventricular systolic dysfunction group was divided into two subgroups: LVEF <50% and (Group 1a) and LVEF 50–54% (Group 1b). The Selvester score was compared between Group 1 and Group 2, and between Group 1a, Group 1b, and Group 2. The predictive role of Selvester score in trastuzumab-induced left ventricular systolic dysfunction was determined with univariate and multivariate analysis.

Results: The mean age of the patients was 56.7±13.7 years. Twenty (21.1%) patients had trastuzumab-induced left ventricular systolic dysfunction. The Selvester score was similar between Group 1 and Group 2. Group 1a had a significantly greater Selvester score compared to Group 1b and Group 2 ($p<0.05$); however, Group 1b and Group 2 had similar Selvester scores ($p>0.05$). The Selvester score was significantly correlated with left ventricular systolic dysfunction in univariate analysis ($r=0.189$, $p<0.05$) but not in multivariate analysis.

Conclusion: Selvester score may be useful especially for detecting severe trastuzumab-induced left ventricular systolic dysfunction.

Keywords: Breast cancer, heart, selvester score, systolic dysfunction, trastuzumab

INTRODUCTION

Breast cancer is the most common cancer in women, and it is associated with the highest number of cancer-related deaths in the same gender (1). Although survival rates have been recently improved by surgery and chemotherapy regimens, the need for novel therapies remains. Trastuzumab is a monoclonal antibody against HE-2 protein and has a confirmed efficacy against metastatic and early breast cancers overexpressing HER-2 as monotherapy or in combination with other agents (2-7). Cardiotoxicity, particularly left ventricular systolic dysfunction, is the most important side effect of trastuzumab (8). Trastuzumab-induced cardiotoxicity has been described with monotherapy and/or combination therapy (9), with the risk particularly being particularly increased by a history of anthracycline use (10, 11). Trastuzumab cardiotoxicity appears to be mediated by the inhibition of the human epidermal growth factor receptor 2, causing ATP depletion and loss of contractile properties and strength (12); however, other mechanisms involving immune destruction of cardiomyocytes have also been described (12, 13).

Transthoracic echocardiography plays a central role in determining trastuzumab-induced left ventricular systolic dysfunction. However, it is operator dependent, costly, and time-consuming. Moreover, it is not always available, particularly in the emergency setting. Therefore, simpler tools are needed to determine trastuzumab cardiotoxicity. Twelve-lead electrocardiography (ECG) is a quick, relatively easy-to-interpret, cheap, and widely available non-invasive tool. It is less operator dependent, and has a high

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reproducibility. The Selvester score is a 12-lead ECG tool to indicate left ventricular infarct size and extent, particularly in the case of myocardial infarction (14). The original Selvester score is composed of 54 criteria scored with a total of 32 points. A modified, simplified form of the Selvester score was developed later, consisting of 50 criteria with a total score of 31 points (15, 16). ECG criteria in Selvester score include wave durations (Q or R), wave amplitude (R or S), and amplitude ratios (R/Q or R/S). The original score's each point roughly corresponds to 3% of left ventricular mass/volume, and the score provides the closest ECG approximation to infarct and scar volume of the left ventricle (16, 17). To date, the role of the Selvester score has been shown for a number of disorders and conditions characterized by ventricular scarring, such as ischemic and non-ischemic cardiomyopathy (18, 19), Chagas disease (20), and acute myocardial infarction (21). Nevertheless, to the best of our knowledge, the Selvester score has not been studied previously among breast cancer patients who were administered trastuzumab and developed left ventricular systolic dysfunction. Herein, we aimed to assess the Selvester score in 12-lead ECG in detecting left ventricular systolic dysfunction among breast cancer patients who received trastuzumab.

METHODS

Başkent University Institutional Review Board and Ethics Committee approved this study and Başkent University Research Fund supported the study (Project No: KA16/57). No informed consent was obtained from the patients since this was a retrospective study performed with screening of medical records. The patients who received trastuzumab between 01.01.2011 and 16.02.2016 were determined from among 726 patients who presented to our hospital's division of medical oncology and diagnosed with breast cancer. A total of 130 patients that received trastuzumab were identified. All the patients underwent serial clinical and echocardiographic cardiac examination after trastuzumab initiation. All the patients were examined by echocardiography after each trastuzumab course by expert echocardiographer physicians using a General Electric Vivid E9 (Horten, Norway) Echocardiography device. Left ventricular systolic dysfunction was considered as a left ventricular ejection fraction (LVEF) drop of at least 5% with a final LVEF dropping below 55% in symptomatic patients or a LVEF drop of at least 10% with a final LVEF dropping below 55% in asymptomatic patients (8). The patients were deemed to have trastuzumab-induced left ventricular systolic dysfunction anytime when they were detected to have a drop in LVEF compared to baseline, as described above. Patients with previous left ventricular systolic dysfunction (LVEF<55%) and those with left ventricular systolic dysfunction attributable to other causes (acute myocardial infarction, cardiac ischemia, myocarditis, toxins, etc.) were excluded. Additionally, patients with left or right bundle branch block, left anterior or posterior fascicular block, left or right ventricular hypertrophy, preexcitation, low voltage, or ventricular pacing on 12-lead ECG were excluded. The Selvester score was calculated using standard 12-lead ECGs taken with the patient in supine position. The Selvester score was composed of 38 ECG criteria rated by 22 points, excluding the posterior V1 and V2 derivations included in the original score (Table 1) (22).

Table 1. The 38–criteria, 22–point, modified Selvester QRS–scoring system excluding the posterior V1 and V2 leads. If ≥2 criteria within the same gray–shaded box were met, only the criterion generating the highest number of points was considered. In each lead, the maximum lead score is seen within parenthesis

Lead	Criterion	Points
I (1)	Q≥30 ms	1
	R/Q≤1	1
	R≤0.2 mV	1
II (2)	Q≥40 ms	2
	Q≥30 ms	1
aVL (2)	Q≥30 ms	1
	R/Q<1	1
aVF (5)	Q≥50 ms	3
	Q≥40 ms	2
	Q≥30 ms	1
	R/Q≤1	2
	R/Q≤2	1
V1 (1)	Any Q	1
V2 (1)	Any Q	1
	R<RV1	1
	R≤10 ms	1
	R≥0.1 mV	1
V3 (1)	Any Q	1
	R≥20 ms	1
	R≥0.2 mV	1
V4 (3)	Q≥20 ms	1
	R/Q≤0.5	2
	R/S≤0.5	2
	R/Q≤1	1
	R/S≤1	1
	R≤0.7 mV	1
V5 (3)	Q≥30 ms	1
	R/Q≤1	2
	R/S≤1	2
	R/Q≤2	1
	R/S≤2	1
	R≤0.7 mV	1
V6 (3)	Q≥30 ms	1
	R/Q≤1	2
	R/S≤1	2
	R/Q≤3	1
	R/S≤3	1
	R≤0.6 mV	1

V: Chest electrodes (V1–V6); Q: Q wave in QRS complex; R: R wave in QRS complex; S: S wave in QRS complex; aVL: stands for augmented unipolar limb lead in which the positive electrode is on the left arm. aVF: stands for augmented unipolar limb lead in which the positive electrode is on the right leg.

The patients were divided into two main groups on the basis of left ventricular systolic dysfunction (Group 1, left ventricular systolic dysfunction present; and Group 2, left ventricular systolic dysfunction absent). Patients with left ventricular systolic dysfunction were also divided further into subgroups of more severe left ventricular systolic dysfunction (LVEF <50%) and less severe left ventricular systolic dysfunction (LVEF 50–54%). Group 1 and Group 2 were compared regarding the Selvester score on 12-lead ECG; this was followed by the comparison of Group 1a, Group 1b, and Group 2 for the same variable. The Selvester score was also assessed in univariate and multivariate analyses to seek its correlation with left ventricular systolic dysfunction.

Statistical Analysis

IBM Statistical Package for the Social Sciences Statistics Version 20.0 (SPSS IBM Corp.; Armonk, NY, USA) software package was used to perform the statistical analyses. Descriptive statistics included mean and standard deviation for normally distributed quantitative variables; median (minimum-maximum) for non-normally distributed continuous variables; and number (frequency) for categorical variables. The Kolmogorov-Smirnov test was used to test the distribution of quantitative variables. The normally distributed quantitative variables were compared between Group 1 and Group 2 using Student's t test and the non-normally distributed continuous variables were compared with the Mann Whitney-U test. Three-group comparisons between Group 1a, Group 1b, and Group 2 were performed with the Kruskal Wallis test, and when the comparison was statistically significant, the Mann Whitney-U test was used to determine the significantly different pair(s). Categorical variables were compared with the Chi-square test. Correlation analyses were performed with Pearson correlation analysis or Spearman correlation analysis, depending on data distribution. Multivariate analysis of left ventricular systolic dysfunction was performed with Binary Logistic Regression Analysis to determine the independent predictors of left ventricular systolic dysfunction.

RESULTS

Among 726 patients who presented to Başkent University Faculty of Medicine, Department of Internal Medicine Division of Medical Oncology between 01.01.2011 and 16.02.2016, a total of 130 patients received trastuzumab therapy. Among these, 70 patients were excluded due to missing ECG data or meeting the exclusion criteria. Of the remainder 60 patients, all (100%) patients were female. The mean age was 56.7±13.7 years. The breast cancer subtypes were ductal carcinoma in 42 (70.0%) patients, lobular carcinoma in 6 (10.0%) patients, and other types (mucinous, tubular, medullary, or papillary) in 10 (16.7%) patients. Twenty-one (36.0%) patients had metastatic disease; 48 (50.0%) patients were operated; and 26 (43.3%) received radiotherapy. While 8 (13.3%) patients did not receive any previous line of chemotherapy, 25 (41.7%) had been administered anthracyclines and 27 (45.0%) other chemotherapy agents. Table 2 presents the demographic and clinical parameters of the general study population.

A total of 20 (15.4% of the original trastuzumab population and 33.3% of the final study population) patients developed trastuzumab-induced left ventricular systolic dysfunction. Of these, 14 (70%) were asymptomatic and 6 (30%) were symptomatic. Among 20 patients developing trastuzumab-induced left ventricular systolic dysfunction, 9 (45%) had a LVEF of <50% (Group 1a) and 11 (55%) had an LVEF of 50–54% (Group 1b). Those in Group1, 12 (60%) patients had persistent LVEF drop and 8 (40%) patients had reversible LVEF drop. All the patients in Group 1a

Table 2. Demographic properties of the whole study population (n=60)

Property	Mean±SD; Frequency (n, %)
Age (years)	56.7±13.7
Female sex	60 (100%)
Carcinoma type	
Ductal	42 (70.0%)
Lobular	6 (10%)
Other (mucinous, tubular, medullary, papillary, etc.)	10 (16.7%)
Operation history	48 (80%)
Previous lines of chemotherapy	
Anthracycline	25 (41.7%)
Other	27 (45.0%)
None	8 (13.3%)
Radiotherapy history	26 (43.3%)
Metastatic disease	21 (36.0%)
Hypertension	22 (36.7%)
Smoking	12 (20.0%)
Alcohol (more than two standard drinks per day)	2 (3.3%)
Diabetes mellitus	11 (18.3%)
Coronary artery disease	7 (11.7%)
Hyperlipidemia	11 (18.3%)
Family history of cardiomyopathy	1 (1.7%)
Cardioactive medications	
Beta blocker	8 (13.3%)
Calcium channel blocker (non–dihydropyridine)	3 (5.0%)
ACE inhibitor/ARB	13 (21.7%)
Spironolactone	2 (3.3%)
Digoxin	2 (3.3%)
Antiplatelet agents	13 (21.7%)
Statin	11 (18.3%)

ACE: angiotensin converting enzyme; ARB: angiotensin receptor blocker

Table 3. Comparison of demographic, treatment and clinical parameters between the study groups with vs without systolic left ventricular dysfunction

Parameter	Systolic left ventricular dysfunction present (n=20)	Systolic left ventricular dysfunction absent (n=40)	p
Age (years)	55.1±14.6	57.0±12.3	NS
Female sex	20(100%)	40 (100%)	NS
Carcinoma type			
Ductal	13 (65%)	29 (72.5%)	NS
Lobular	2 (10.0%)	4 (10.0%)	NS
Other (mucinous, tubular, medullary, papillary, etc.)	3 (15.0%)	7 (17.5%)	NS
Operation history	15 (75.0%)	32 (80.0%)	NS
Previous lines of chemotherapy			
Anthracycline	12 (60.0%)	13 (32.50%)	<0.05
Other	8 (40.0%)	19 (47.5%)	NS
Radiotherapy history	8 (40.0%)	18 (45.0%)	NS
Metastatic disease	6 (30.0%)	15 (37.5%)	NS
Hypertension	10 (50.0%)	12 (30.0%)	<0.05
Smoking	5 (20.0%)	7 (17.5%)	NS
Alcohol (more than two standard drinks per day)	1 (5.0%)	1 (2.5%)	NS
Diabetes mellitus	4 (20.0%)	7 (17.5%)	NS
Coronary artery disease	2 (10.0%)	5 (12.5%)	NS
Hyperlipidemia	4 (20.0%)	7 (17.5%)	NS
Family history of cardiomyopathy	0 (0%)	1 (2.5%)	NS
Cardioactive medications			
Beta blocker	3 (15.0%)	5 (12.5%)	NS
Calcium channel blocker (non-dihydropyridine)	1 (5.0%)	2 (5.0%)	NS
ACE inhibitor/ARB	4 (20.0%)	9 (22.5%)	NS
Spironolactone	1 (5.0%)	1 (2.5%)	NS
Digoxin	1 (5.0%)	1 (2.5%)	NS
Antiplatelet agents	4 (20.0%)	9 (22.5%)	NS
Statin	4 (20.0%)	7 (17.5%)	NS

ACE: angiotensin converting enzyme; ARB: angiotensin receptor blocker

(n=9) had persistent LV dysfunction while three (27.3%) of patients in the mild LV dysfunction had persistent LV dysfunction while 8 (72.7%) of them had reversible LV dysfunction, that is all patients with reversible LV dysfunction were in the mild LV dysfunction group. A comparison of Group 1 and Group 2 revealed that a history of anthracycline chemotherapy and hypertension were significantly more common in patients with trastuzumab-induced left ventricular systolic dysfunction than those without (for both, p<0.05) (Table 3); the other demographic and clinical parameters were similar between the two groups. A comparison of the Selvester score between Group 1 and Group 2 revealed no significant difference regarding trastuzumab-induced left ventricular systolic dysfunction (5.83±1.11 vs 4.89±1.19 p>0.05). A comparison of Group 1a, Group 1b, and Group 2, on the other hand, revealed that Group 1a showed a significantly greater Selvester score than Group 1b and Group 2 did (7.29±1.61 vs 4.63±1.47; p<0.05 and 7.29±1.81 vs 4.89±1.29, respectively; p<0.05). However, there was no significant differ-

ence between Group 1b and Group 2 regarding the Selvester score (p>0.05). In the comparison of the Selvester score within the reversible LV dysfunction group (n=8), no significant difference was noted between the Selvester scores at the time of LV dysfunction and after recovery of LV dysfunction (5.13±1.29 vs 4.99±1.78, p>0.05) The Selvester score was significantly, albeit only moderately strongly, correlated to trastuzumab-induced left ventricular systolic dysfunction (r=0.189, p<0.05). A multivariate analysis with binary logistic regression showed that the Selvester score was not significantly and independently correlated to trastuzumab-induced left ventricular systolic dysfunction (χ^2=1.14; %95 GA 0.92–1.26, p>0.05).

DISCUSSION

Breast cancer is the most common cancer type that is responsible for the most cancer-related deaths among women worldwide (1). It has been reported that HER2-positive (HER2+) subtypes constitute 20% of all breast cancer types and are related to poor prognosis. However, with the advent of anti-HER2 targeted therapies, this has started to change. Trastuzumab is a monoclonal antibody that binds to the extracellular domain of HER2 and acts via activation of antibody-dependent cytotoxicity, inhibition of signal conduction, inhibition of neoangiogenesis, and inhibition of repair of treatment-induced DNA injury (23*)*. Trastuzumab is used alone or in combination as neoadjuvant, adjuvant therapies, and in metastatic disease (24). Cardiotoxicity is the most important side effect of trastuzumab, which is most commonly seen as asymptomatic LVEF depression or, less commonly as overt congestive heart failure (8*)*. Available data about trastuzumab cardiotoxicity is limited, and various mechanisms, including angiotensin II upregulation and resulting cell death due to oxidative stress (24), antiapoptotic protein BCL-XL's downregulation and the proapoptotic protein BCL-XS's upregulation (25) and inability of maintaining sarcomeres' structure and function and dysregulated scavenging mechanism for the proapoptotic oxidative subproducts (26, 27) have been postulated.

Trastuzumab's cardiotoxicity is shown by a variety of echocardiographic methods, of which biplane disks method (modified Simpson's rule) is the most commonly used one, as in our study. Additionally, M-Mode echocardiography, three dimensional echocardiography, speckle tracking echocardiography, and deformation imaging (strain and strain rate), as well as cardiac magnetic resonance imaging, are also utilized (28-30). However, most of the above-mentioned techniques are technologically demanding and time-consuming, and associated with increased cost and limited availability. Moreover, most echocardiographic techniques are operator dependent and flawed by a significant inter-observer variability in LVEF measurement. Therefore, simpler, cheap, readily available techniques are needed to assess left ventricular systolic function in trastuzumab-treated patients. Twelve-lead ECG is a simple, rapidly and widely available test relatively independent of operator interpretation. Therefore, it may offer promise in detecting trastuzumab-induced left ventricular systolic dysfunction.

The Selvester score is a 12-lead ECG score that indicates left ventricular infarct size and extent (14). The original Selvester score is composed of 54 ECG criteria with a total of 32 points. The subsequently developed modified Selvester score consists of 50 criteria with a total of 31 points (15, 16). The Selvester score represents the best electrocardiographic approximation of infarct volume (17). Its role has been shown in a number of disorders and clinical scenarios characterized by myocardial scarring, including ischemic and non-ischemic cardiomyopathy (18, 19), Chagas disease (20), and acute myocardial infarction (21). However, as far as we know, the Selvester score has not been studied in breast cancer patients with trastuzumab-induced left ventricular systolic dysfunction.

In our study, the modified Selvester score did not show any statistically significant difference between the study groups with and without trastuzumab-induced left ventricular systolic dysfunction (Group 1 and Group 2, respectively). However, the patient subgroup with more severely depressed LVEF (LVEF<50%) (Group 1a) had a significantly greater Selvester score compared to both less severely affected patients (LVEF 50–54%) (Group 1b) and the controls (Group 2). These results suggest that the Selvester score was significantly increased among patients with more severe left ventricular systolic dysfunction, who possibly had myocardial scarring. Unfortunately, we had no MRI data to show myocardial scarring in such patients. However, 12 (60%) of those who had LVEF drop had a persistent LVEF drop, and these patients encompassed all the patients in Group 1a (more severe trastuzumab left ventricular systolic dysfunction). Furthermore, all the patients with reversible LV dysfunction had mild LV dysfunction. These findings suggests that as more severe left ventricular systolic function occurs, more scarring or persistent myocardial fibrosis occurs, increasing the Selvester score. This finding is in line with the previous observations where the Selvester score was closely related to myocardial scar after myocardial infarction (14). In fact, the original definition of trastuzumab-induced left ventricular systolic dysfunction, that is a 10% LVEF drop to below 55% in asymptomatic patients or a 5 percent LVEF drop to below 55% in symptomatic patients, may not necessarily show severe left ventricular injury in every patient. Some patients may temporarily develop such dysfunction, and some due to contractile dysfunction rather than scarring. Additionally, echocardiography, in its all forms, is heavily operator dependent and, thus, may show LVEF variability. In relation to this information, patients with mild LVEF drop may in fact be included as depressed LVEF, which approximated the Selvester scores in both groups, precluding any significant difference between the patient and control groups regarding the Selvester score. However, as subgroup analyses showed a significantly greater Selvester score in patients with more severely depressed LVEF (LVEF<50%), those patients may have true LVEF depression. One may speculate that ECG changes may not occur without, or with small amount of, scarring or fibrosis in the left ventricular myocardium. The Selvester score, as a constellation of ECG parameters, may actually be expected to better delineate any left ventricular systolic dysfunction than single ECG parameters like isolated Q waves, loss of R wave height, or persistent ST elevation. The lack of statistical significance between patients with and without left ventricular systolic dysfunction may have resulted from a lower number of patients with more severely depressed LVEF, thus, limiting our statistical power.

A reduction of the Selvester score in patients with reversible trastuzumab-induced LV dysfunction after normalization of LV function would be a plausible finding. However, when we re-calculated the Selvester score after normalization of LV function and compared it with the initial Selvester score among patients with LV dysfunction, we identified no significant difference between the two scores. This was interpreted in three ways: First, the low number of subjects in reversible LV dysfunction subgroup masked a statistically significant change in the Selvester score; second, the Selvester scores may be similar in patients with and without subtle degrees of LV dysfunction. This hypothesis was indeed corroborated by the lack of statistical significance between the initial Selvester scores of control subjects and those with a minor LV dysfunction; third, some minor degree of LV dysfunction (LVEF 50–54%) may have actually been a measurement difference between independent echocardiographers performing the echocardiographic studies, and, thus, there may have not been true LV dysfunction and/or normalization at all in these patients. Therefore, we could not ascertain the exact role of the Selvester score in reversible trastuzumab-induced LV dysfunction. This issue should be addressed in a study prospective randomized, controlled study.

Our study has some limitations. First, this was a retrospective study with a relatively small study sample. Second, we could not assess posterior ECGs and, therefore, excluded posterior ECG criteria of the ECG leads V1 and V2, which limited the sensitivity of the study. Third, we did not exclude coronary artery disease as the cause of left ventricular systolic dysfunction. However, we determined trastuzumab-induced left ventricular systolic dysfunction on the basis of temporal and clinical features, with coronary artery disease being unlikely in our patients considering the lack of ischemic symptoms, gender (all female), and age (predominantly of middle age) of the study population.

CONCLUSION

The Selvester score assessed from 12-lead ECG may be indicative of severe left ventricular systolic dysfunction evidenced by subnormal LVEF (<50%) among patients receiving trastuzumab therapy. However, patients with less severe left ventricular systolic impairment may not be detected by the Selvester score as they may have no scarring in the left ventricular myocardium but only reversible systolic dysfunction. Further large-scale studies are needed to answer these questions.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Başkent University (Approval Date: 09/02/2016; Approval No: KA16/57).

Informed Consent: As the present study had a retrospective design, no written/verbal consent was obtained from the patients.

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Original Research

Can the Prognosis of Diffuse Large B-Cell Lymphoma be Predicted by a Simple CBC Count?

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ABSTRACT
Objective: In this study, we aimed to develop a new prognostic model using the neutrophil-to-lymphocyte ratio (NLR) and defined prognostic indexes to improve the results in patients with diffuse large B-cell lymphoma (DLBCL).
Methods: The data of 340 newly diagnosed patients with DLBCL, who underwent at least two cycles of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), were evaluated retrospectively. A receiver operating characteristic (ROC) curve analysis was used to determine the NLR cut-off value. The NLR cut-off value was 4.76. In the study, a total of 231 patients (67.9%) were in the low NLR ≤4.76 group, while 109 patients (32.1%) were in the high NLR (NLR >4.76) group.
Results: The 5-year overall survival (OS) was 37.1%, and 78.9% in the high NLR group, and low NLR group, respectively. A high pre-treatment NLR was associated with a worse OS and progression-free survival (PFS) (both p<0.001, respectively). In the multivariate analysis, a high pre-treatment NLR and a high National Comprehensive Cancer Network-International Prognostic Index (NCCN-IPI) status were found to be as an independent risk factors of poor OS (hazard ratio [HR]= 2.28; 95% confidence interval [CI]= 1.51-3.45; p= 0.001; HR= 5.59; 95% CI= 3.22-9.70; p= 0.001, respectively) and PFS.
Conclusion: The study found that a high NLR was associated with a poor treatment response, poor PFS, and OS. In view of these data, we believe that the creation of an inflammation-based cumulative prognostic score system (IBCPSS), by adding NLR among the factors whose prognostic importance has been proven in DLBCL, can especially shed light on the early diagnosis of patients with a poor prognosis, aggressive treatment decision, and individualization of treatment.
Keywords: Diffuse large B-cell lymphoma, score system, neutrophil-to-lymphocyte ratio, prognosis

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphomas, and it accounts for 40% of all lymphomas (1). As a result of adding rituximab to a combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), a dramatic improvement was seen in the treatment outcome, progression-free survival (PFS), and overall survival (OS) (2). However, resistance to the initial treatment regime and post-treatment recurrence are seen in over 30% of DLBCL patients (3). The IPI is a risk classification system commonly used to predict the outcome and choose the best therapeutic treatment in patients with aggressive lymphoma (4). However, IPI is insufficient, particularly in the identification of patients with a poor prognosis and low probability of survival, as it was established in the pre-ritumixab era. Therefore, IPI capacity enhancement studies have been carried out, and IPI variants such as revised IPI (R-IPI) (5) and, the National Comprehensive Cancer Network-IPI (NCCN-IPI) (6) have been developed in risk classification. Unfortunately, these models are also insufficient to identify patients with a poor prognosis who cannot benefit from the R-CHOP treatment. Additionally, it is known that an adequate survival benefit could still not be reached in a significant number of patients with DLBCL despite the addition of rituximab to the CHOP treatment (7). Therefore, studies are still underway to identify new markers for the prognosis in patients with DLBCL receiving R-CHOP therapy. In addition to the conventional prognostic factors in the literature, the importance of some inflammatory aspects in the prognosis of DLBCL has been investigated. A high C-reactive protein (CRP) level (8), lower absolute lymphocyte-to-monocyte ratio (LMR) (9), high Beta-2 microglobulin level (B2-MG) (10), and a high absolute neutrophil count (ANC) (11) were associated with poor prognosis, while a high absolute lymphocyte count (ALC) (12) was defined as a positive prognostic factor in response to treatment. Prognostic importance of the plasma fibrinogen level in DLBCL could not be shown (13). It was reported that there was a close link between the development of lymphoma, and chronic inflammation, immunodeficiency, and infections. In addition, the role of immune response and abnormal inflammatory response in predicting the clinical course in patients has also been shown in some studies (14). The neutrophil-to-lymphocyte ratio (NLR) is an important parameter that indicates a systemic inflammatory response, and provides an advantage in the determination of prognosis when compared to ALC and ANC. It has been proven that it could be used as an important predictor for survival estimation in many cancers (15, 16). It was first reported by Porrata et al. (17) as an independent prognostic factor in patients with DLBCL treated with R-CHOP. The prognostic significance of NLR

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in DLBCL was then demonstrated in a limited number of studies (18, 19). The aim of this study was to evaluate the prognostic and predictive value of NLR in patients with DLBCL treated with R-CHOP as the first-line therapy together with clinical and laboratory parameters to make a new model to improve the well-known prognostic index.

METHODS

Patients

The data of 340 newly diagnosed patients with DLBCL, who were followed up in the Gaziantep University of Hematology Department between 2004 and 2018, and underwent at least two cycles of R-CHOP in the first-line treatment, were evaluated retrospectively. Patients who did not receive R-CHOP as the first-line therapy and had an active infection at the time of diagnosis were excluded from the study. The study was approved by Gaziantep University Medical Faculty Medical Ethics Committee, which was dated 23.01.2019 and numbered 2019/40, and a written informed consent was obtained.

Clinical Data

Patients' clinical parameters (age, gender, Eastern Cooperative Oncology Group performance score [ECOG-PS], B symptoms, bulky disease, stage, extranodal disease), laboratory parameters (serum lactate dehydrogenase [LDH], B2-MG, CRP levels and albumin value, erythrocyte sedimentation rates [ESR], NLR, treatment response, and risk classification (IPI, R-IPI, NCCN-IPI) were carefully recorded at the time of diagnosis from the patient files. The neutrophil, and lymphocyte counts were determined from routine complete blood count obtained at the time of diagnosis of NHL using Sysmex automated hematology analyzers (Sysmex XN 9000, Erlangen, Germany). The cut-off value for laboratory parameters was determined on the basis of the upper/lower limit of the local laboratory, and the cut-off point for NLR was found to be 4.76 ($p<0.001$; AUC= 0.686; Sensitivity= 56.14% [95% CI= 46.50-65.40]; Specificity= 80.09% [95% CI= 74.30-85.10] using the receiver operating characteristic (ROC) analysis.

Statistical Analysis

A chi-square test was performed in the comparison of characteristics of the high and low NLR groups. The Kaplan-Meier method was used to calculate PFS and OS, and a log-rank test was used to compare PFS and OS lifespan according to the NLR. Univariate and multivariate survival analyses were calculated using the Cox' regression model. A p-value <0.05 was considered statistically significant. Count, percentage, mean, and standard deviation values of the data were calculated. The Statistical Package for the Social Sciences 22.0 (SPSS IBM Corp.; Armonk, NY, USA) program was used in the analysis of all data.

RESULTS

Associations of NLR with Clinical Characteristics

Of the 340 patients included in the study, 154 were females, 186 were males, and the mean age was 52.71±16.85 years. Characteristics of patients in the high and low NLR groups are summarized in Table 1. A total of 114 (33.5%) of the patients included in the study was died due to refractory/relapse lymphoma. There were 231 (67.9%) and 109 (32.1%) patients in the high and low NLR groups, respectively. There were significant differences between the high and low NLR groups in terms of patient characteristics. When comparing the patients in the high NLR group with those in the low NLR group, the high NLR group was significantly correlated with an advanced age (>60 years; 48.7% vs. 51.3%, $p<0.001$), poor performance status (ECOG-PS 2-3, 73.6% vs. 26.4%; $p<0.001$), high LDH (normal LDH >3, 64.3% vs. 35.7%, $p<0.001$), high ESR (ESR >40, 44.6% vs. 55.4%, $p<0.001$), high B2-MG level (B2-MG ≥3.5, 55.5% vs. 44.5%, $p<0.001$), high CRP level (CRP >5, 40.0% vs. 60.0%, $p<0.001$), low albumin level (albumin <4.5, 34.9% vs. 65.1%, $p<0.001$), and the presence of extranodal sites involvement (extranodal disease, 36.9% vs. 63.1%, $p<0.001$). The NLR was also relevant to bulky disease and B symptoms (both $p<0.001$). There was no significant difference between the high and low NLR groups in terms of the patients' stage at the time of diagnosis ($p=0.636$). Patients in the high NLR group were significantly correlated with a high IPI, R-IPI, and NCCN-IPI (high-intermediate to high IPI, 50.0% vs. 50.0%, $p<0.001$; poor R-IPI, 49.0% vs. 51.0%, $p<0.001$; high-intermediate to high NCCN-IPI, 46.7% vs. 53.3%, $p<0.001$).

Treatment Response

The treatment response of patients who received R-CHOP as the first-line therapy was grouped as complete response (CR), and partial response, stable disease, no response, and progressive disease. A 25% dose reduction was made in the treatment regime of a limited number of patients over the age of 75, excluding rituximab. CR rate was lower in the high NLR group as compared to the low NLR group (24.6% vs. 75.4%; $p<0.001$; Table 1).

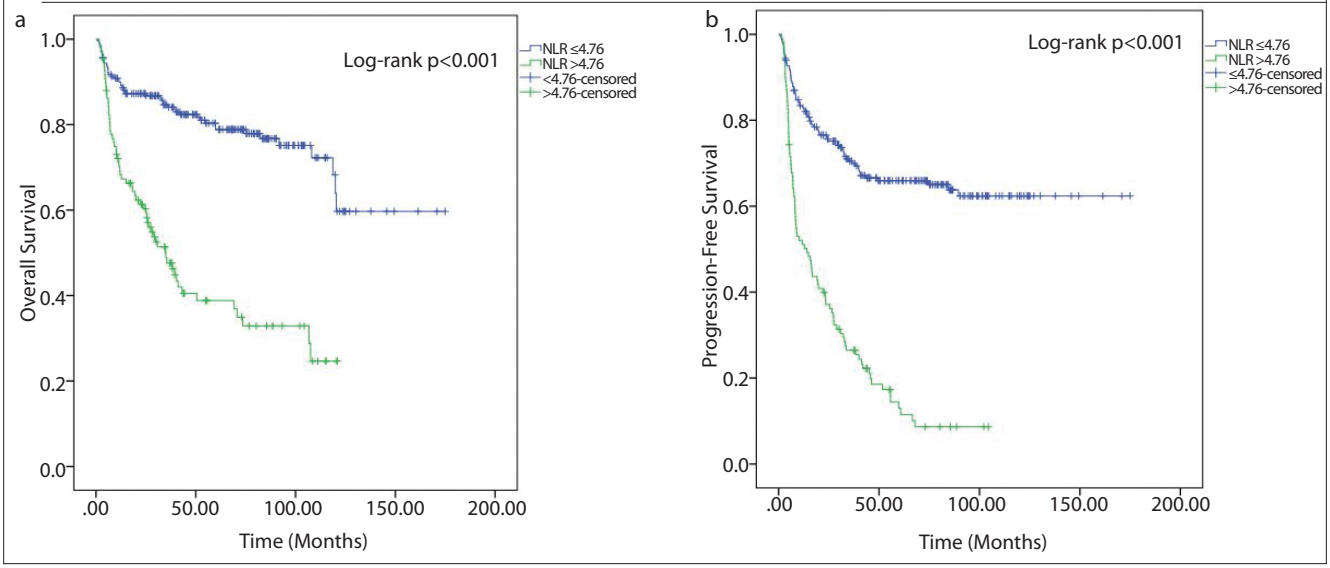
Survival

The median follow-up time was 38.5 months (range, 0.2-170 months). The 5-year OS and 5-year PFS rates were 66.4% and 48.5%, respectively. The 5-year OS rate was 37.1%, and 78.9% in the high NLR group, and low NLR group, respectively. A high pre-treatment NLR was found to be associated with poor OS (the mean OS for NLR >4.76: 53.95±5.05; 95% CI= 44.03-63.87; $p<0.001$; Figure 1a). The 5-year PFS rate, on the other hand, was 13.0%, and 65.5% in the high NLR group, and low NLR group, respectively. A high pre-treatment NLR was associated with poor PFS (the mean PFS for NLR >4.76: 26.82±2.98; 95% CI= 20.97-32.67; $p<0.001$; Figure 1b).

The Cox regression model was used to determine the important variables in the PFS and OS times. In the Cox regression analysis, variables with a high level of correlation between each other (age, ECOG, IPI, R-IPI) were checked for their Variance inflation factor coefficient and excluded from the analysis. On univariate analysis, an elevated LDH, extranodal sites involvement, and high NLR and high NCCN-IPI scores were significantly associated with poor OS (HR=1.97, 95% CI=1.23 - 3.14, p=0.004; HR= 2.34, 95% CI=1.52 - 3.58, p=0.001; HR=3.88, 95% CI=2.67 - 5.65, p=0.001; HR=8.12, 95% CI=4.90 - 13.45, p=0.001, respectively). In a multivariate analysis, only pre-treatment NLR and NCCN-IPI remained as independent prognostic factors (HR=2.28, 95% CI=1.51 - 3.45, p=0.001; HR=5.59, 95% CI=3.22 - 9.70, p=0.001, respectively; Table 2).

Figure 1. a, b. Survival (a) OS and (b) PFS According to NLR

NLR: neutrophil-to-lymphocyte ratio; OS: overall survival; PFS: progression-free survival



A high LDH, extranodal disease, high NLR, and high NCCN-IPI scores at the time of diagnosis were associated with a poor PFS in the univariate analysis (HR=1.78, 95% CI=1.23 - 2.58, p=0.002; HR=2.08, 95% CI=1.49 - 2.92, p=0.001; HR=4.18, 95% CI=3.06 - 5.69, p= 0.001; HR=3.05, 95% CI=2.21 - 4.21, p=0.001, respectively; Table 3). A high pre-treatment LDH, extranodal disease, high NLR, and high NCCN-IPI status were found to be also significant in the multivariate analysis, and as independent risk factors for poor PFS (HR=1.87, 95% CI=1.23 - 2.85, p=0.003; HR=1.52, 95% CI=1.07 - 2.17, p=0.019; HR=2.93, 95% CI=2.08 - 4.11, p=0.001; HR=1.79, 95% CI=1.24 - 2.59, p=0.002, respectively; Table 3).

DISCUSSION

In the literature, many studies have shown that there is a correlation between a pre-treatment NLR and survival in solid tumors (20, 21). There are a limited number of studies that examine the prognostic importance of NLR in DLBCL, which is the most commonly seen lymphoma subtype among hematologic malignancies. Similar to the studies in the literature, our study showed that a high pre-treatment NLR is associated with a high LDH level, extranodal sites involvement, and poor IPI and NCCN-IPI (18, 22). A significant correlation was demonstrated between a high NLR and poor prognostic indicators such as an advanced age, poor ECOG-PS status, bulky disease and B symptoms, ESR, CRP, B2-MG, and albumin values, which are the indicators of a systemic inflammatory response. Prognostic risk indexes, IPI in the pre-rituximab era, and R-IPI and NCCN-IPI after rituximab were found to be significantly associated with NLR. Only NCCN-IPI and NLR were independent risk factors for survival in the multivariate analysis, while the LDH, extranodal sites involvement, NLR, and NCCN-IPI were found to be independent risk factors in PFS. In this study, the 5-year OS was 37.1% in the high NLR group. Similarly, in the study by SI Go et al. (19) using NCCN-IPI, the 5-year OS was 30%, and in another study, it was 46% (23). Different results in similar studies might be associated with the absence of a specific cut-off value for NLR. In our study, the cut-off value for the pre-treatment NLR was determined as 4.76 using the ROC analysis. The cut-off value varies between 3.0 and 6.0 in different studies (9, 23, 24). There is no ideal method for determining the NLR cut-off value. This limits the value of our study as it did in similar previous studies. However, it seems possible to increase the prognostic value of NLR by determining a standardized cut-off value and evaluating the NLR together with parameters whose prognostic importance has been proven to make an individualized risk classification. Despite the different results, the effect of NLR on survival has been reported in many studies (17, 24). The potential mechanism is still not exactly known. However, some possible mechanisms have been defined. First, circulating interleukin (IL)-17 and IL-18 levels were found to be elevated in the serum of patients with a high NLR (25). These proinflammatory cytokines have been shown to play a role in the continuity of the tumor microenvironment and the aggressive course of the tumor (26). Second, a high NLR reflects increased neutrophil count, and a high neutrophil count is associated with the vascular endothelial growth factor synthesis, which plays a vital role in tumor development and angiogenesis (25). The third potential mechanism in the correlation between a high NLR and survival is that a high NLR causes suppression of the ALC. Lymphocytes create a host immune response against malignancies (22). In some immunologic studies performed on solid tumors, it was shown that an increased ANC in the peripheral blood are actually CD11, CD33, and CD15 positive myeloid-derived suppressor cells (MDSC), which play a role in the development and progression of cancer by suppressing the human immune system (lymphopenia). Also, tumor-associated neutrophils (TANs) derived from MDSC have an important role in the tumor proliferation and metastasis. There are two different phenotypes of TANs: protumorogenic and anti-tumorogenic. An increase in tumorogenic TANs leads to a high NLR with T-cytotoxic suppression (27, 28). In this context, it is evident that differential diagnosis of these two phe-

Table 1. Patients' characteristics and treatment response

		NLR		
		≤4.76	>4.76	p
Age	≤60	170 (76.9)	51 (23.1)	<0.001
	>60	61 (51.3)	58 (48.7)	
Sex	Male	125 (67.2)	61 (32.8)	0.816
	Female	106 (68.8)	48 (31.2)	
ECOG - PS	0 - 1	212 (79.1)	56 (20.9)	<0.001
	2 - 3	19 (26.4)	53 (73.6)	
B symptoms	No	184 (81.1)	43 (18.9)	<0.001
	Yes	47 (41.6)	66 (58.4)	
Bulky disease	No	177 (79.4)	46 (20.6)	<0.001
	Yes	54 (46.6)	62 (53.4)	
Stage	1 - 2	89 (66.4)	45 (33.6)	0.636
	3 - 4	142 (68.9)	64 (31.1)	
Extranodal disease	No	101 (75.4)	33 (24.6)	0.024
	Yes	130 (63.1)	76 (36.9)	
LDH	≤1	130 (83.3)	26 (16.7)	<0.001
	<1 to ≤3	76 (66.7)	38 (33.3)	
	>3	25 (35.7)	45 (64.3)	
Response to treatment	CR	190 (75.4)	62 (24.6)	<0.001
	Other	41 (46.6)	47 (53.4)	
IPI	Low to low-intermediate	159 (81.1)	37 (18.9)	<0.001
	High-intermediate to high	72 (50.0)	72 (50.0)	
R-IPI	Very good	37 (90.2)	4 (9.8)	<0.001
	Good	118 (78.1)	33 (21.9)	
	Poor	75 (51.0)	72 (49.0)	
NCCN-IPI	Low to low-intermediate	143 (81.7)	32 (18.3)	<0.001
	High-intermediate to high	88 (53.3)	77 (46.7)	
ESR	<40	154 (76.6)	47 (23.4)	<0.001
	≥40	77 (55.4)	62 (44.6)	
B2-MG	0 - 3.4	174 (82.1)	38 (17.9)	<0.001
	≥3.5	57 (44.5)	71 (55.5)	
CRP	0 - 5	87 (87.0)	13 (13.0)	<0.001
	>5	144 (60.0)	96 (40.0)	
Albumin	0 - 4.4	190 (65.1)	102 (34.9)	0.004
	>4.5	41 (85.4)	7 (14.6)	

ECOG-PS: Eastern Cooperative Oncology Group performance score; LDH: lactate dehydrogenase; CR: complete remission; IPI: International Prognostic Index; R-IPI: Revised-International Prognostic Index; NCCN-IPI: National Comprehensive Cancer Network- International Prognostic Index; ESR: erythrocyte sedimentation rate; B2-MG: Beta-2 microglobulin; CR: C-reactive protein

Table 2. Univariate and multivariate analysis for OS outcomes

	Univariate Analysis		Multivariate Analysis	
	p	HR (95.0% CI)	p	HR (95.0% CI)
Elevated LDH	0.004	1.97 (1.23–3.14)	0.057	1.65 (0.98–2.78)
Ann Arbor stage	0.156	1.32 (0.89–1.96)	0.302	1.23 (0.83–1.82)
Extranodal disease	0.001	2.34 (1.52–3.58)	0.528	1.15 (0.73–1.81)
NLR (≤4.76 vs. >4.76)	0.001	3.88 (2.67–5.65)	0.001	2.28 (1.51–3.45)
NCCN-IPI scores	0.001	8.12 (4.90–13.45)	0.001	5.59 (3.22–9.70)

LDH: lactate dehydrogenase; NLR: neutrophil-to-lymphocyte ratio; NCCN-IPI: National Comprehensive Cancer Network- International Prognostic Index; OS: overall survival

Table 3. Univariate and multivariate analysis for PFS outcomes

	Univariate Analysis		Multivariate Analysis	
	p	HR (95.0% CI)	p	HR (95.0% CI)
Elevated LDH	0.002	1.78 (1.23–2.58)	0.003	1.87 (1.23–2.85)
Ann Arbor stage	0.842	0.96 (0.71–1.31)	0.771	1.04 (0.76–1.43)
Extranodal disease	0.001	2.08 (1.49–2.92)	0.019	1.52 (1.07–2.17)
NLR (≤ 4.76 vs > 4.76)	0.001	4.18 (3.06–5.69)	0.001	2.93 (2.08–4.11)
NCCN-IPI scores	0.001	3.05 (2.21–4.21)	0.002	1.79 (1.24–2.59)

LDH: lactate dehydrogenase; NLR: neutrophil-to-lymphocyte ratio; NCCN-IPI: National Comprehensive Cancer Network- International Prognostic Index; PFS: progression-free survival

notypes cannot be performed by the neutrophil count in the peripheral blood. In addition, a detection of MDSC and TANs in the peripheral blood is a costly method and not practical. It appears to be more appropriate to use NLR as an indicator of proinflammatory state until a new parameter is defined.

It was reported in different studies that ESR (29), CRP (7), and B2-MG (30) can be used to determine the prognosis in DLBCL. This study also showed that the ESR, B2-MG, CRP levels, and the albumin value, which are among the parameters that show a systemic inflammatory response, are associated with high NLR. The search for identifying new prognostic parameters in DLBCL continues. In recent years, the investigation of the prognostic significance of NLR in DLBCL has been a popular study topic among researchers. Despite the different cut-off values, a high pre-treatment NLR was found to be associated with poor survival since it indicates a probable tumor-associated neutrophilia, that is, reflecting a systemic inflammatory response (19, 20). DLBCL is the most commonly seen hematologic malignancy, and the definition of resistance to R-CHOP treatment in a significant number of patients indicates the need for a new risk classification index. In the present study, a pre-treatment NLR was examined together with many proinflammatory parameters, and its effect on OS and PFS was determined. In view of these data, it is believed that the creation of an inflammation-based cumulative prognostic score system (IBCPSS, a new model for risk stratification), by adding NLR in the factors whose prognostic importance has been proven in DLBCL along with ESR, B2-MG, CRP, and albumin levels, which are among the parameters that reflect the systemic inflammatory response, can especially shed light on the early diagnosis of patients with poor prognosis, aggressive treatment decision, and particularly on the individualization of treatment.

As a result, NLR was demonstrated as a strong prognostic marker in patients with DLBCL treated with R-CHOP as the first-line therapy in this study. Also, it was shown that the degree of the systemic inflammatory response plays a major role in the clinical course, treatment response, and predicting prognosis. It seems possible that a new risk scoring system (IBCPSS), which can be established by adapting the inflammation parameters and NLR to the well-known risk classification index, can be used as a more potent prognostic index in the individualized treatment decision in the near future.

Ethics Committee Approval: The study was approved by Gaziantep University Medical Faculty Medical Ethics Committee (dated 23.01.2019 and numbered 2019/40).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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Case Report

Triple Innominate Osteotomy for Early Closure of the Triradiate Cartilage after Hip Joint Injury: A Pediatric Case Report

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ABSTRACT

Acetabular fractures are less common in children than those in adults. Even these fractures are rarely seen in infants and usually such fractures require high-impact trauma. Orthopedic surgeons often recommend conservative treatment for acetabular fractures to accommodate remodeling features. In addition, pelvic osteotomies have demonstrated good results; specifically, triple innominate osteotomy treatment has been proven effective. In this study, we report the case of an 8 year-old girl who suffered from hobbling because of preclosure of triradiate cartilage and hip subluxation with left femoral diaphysis fracture resulting in malunion and lower extremity shortening. First we applied osteotomy and plate and screw osteosynthesis for femoral malunion and afterwards triple innominate osteotomy for subluxation caused by acetabular traumatic dysplasia and preclosure of triradiate cartilage. The results were quite satisfying in this case.

Keywords: Acetabular dysplasia, children, early closure, triple innominate osteotomy, triradiate cartilage injury

INTRODUCTION

Acetabulum fractures in infants are rare, with occurrences estimated at one in a thousand (1). Infant fractures are different from adult fractures. A high-impact trauma is necessary for displaced fractures, and particularly for acetabulum fractures, because of the presence of large amounts of cartilage tissue, thick periosteum, joint elasticity, and ligament force. In infants, pelvic and acetabulum fractures unite easily, with a high percentage of remodeling; thus, physicians generally choose conservative treatment (2-4). Triradiate Cartilage (TRC) is the most important feature of acetabulum fractures in infants, and this feature may be affected during treatment. Furthermore, the acetabulum may stop growing, which can lead to acetabular dysplasia and hip dislocation (5, 6). This study suggested that post-traumatic TRC injuries may be unilateral or bilateral, and that ipsilateral/contralateral joint dislocation and femoral fractures occur when TRC is injured (7-11). When acetabular dysplasia develops, physicians generally choose periacetabular and Chiari osteotomies as the usual surgical treatment.

The aim of this study is to emphasize that examinations of lower extremity injuries resulting from high-impact pediatric trauma should also include a check for pelvic ring injuries, regardless of the rarity with which such injuries occur. Undetected TRC injury may lead to iliac dysplasia. This study emphasizes that triple osteotomy is a good choice for treating post-traumatic acetabular dysplasia and hip subluxation.

CASE PRESENTATION

An 8-year-old girl came to us with a hobbling problem. Her case history indicated a traffic accident when she was one-and-a-half years old, resulting in a left femoral fracture. After the traffic accident, she was treated with a pelvipedal hip spica cast. This problem in the left lower extremity of the patient gradually increased within 6 months after the accident. In this process, her parents had not applied for treatment of this patient in any health care institution.

The physical examination revealed a positive Trendelenburg's sign, and both hip joints' movement capacity was full with her left lower extremity. Spina Iliaca Anterior Superior-medial malleol distance measurements indicated that her left lower extremity was 2.5 cm shorter than her right extremity. Plain radiographies (X Ray Machine; Siemens, Erlangen, Germany) indicated left acetabular dysplasia, left hip subluxation, and left femur rotational and angular malunion (Figures 1a, b). In addition, a computed tomography (CT) scan (Somatom X; Siemens, Erlangen, Germany) of her hips indicated that left TRC had preclosed on all 3 legs of TRC (Figure 1c).

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Figure 1. a-c. Preoperative plain radiographic images and pelvic CT scan. (a) preoperative left femur and pelvis AP radiography. (b) preoperative left femur lateral radiography. (c) preoperative left innominate cartilage arrest showing pelvic CT scan

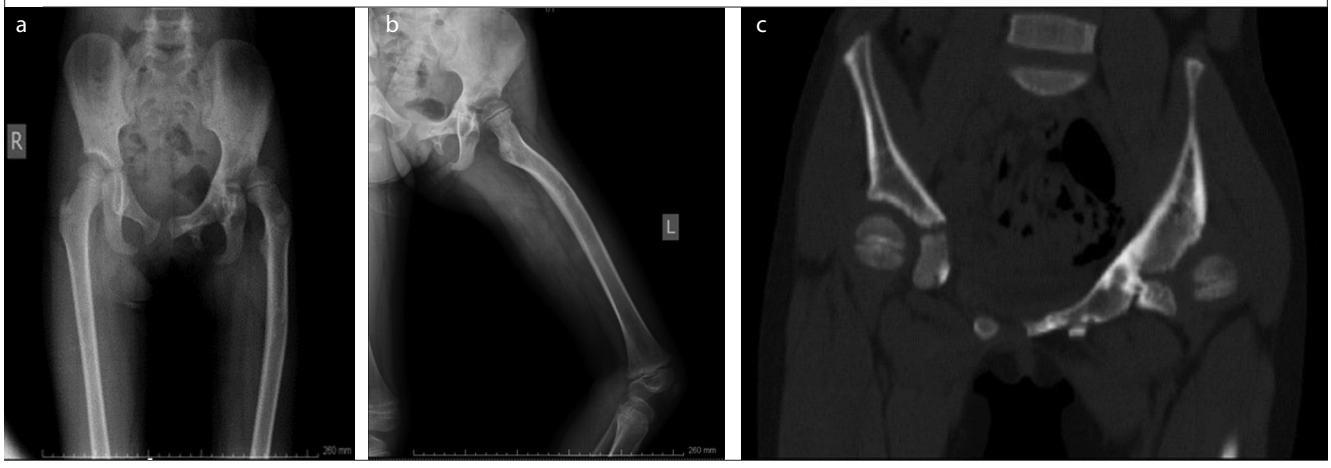
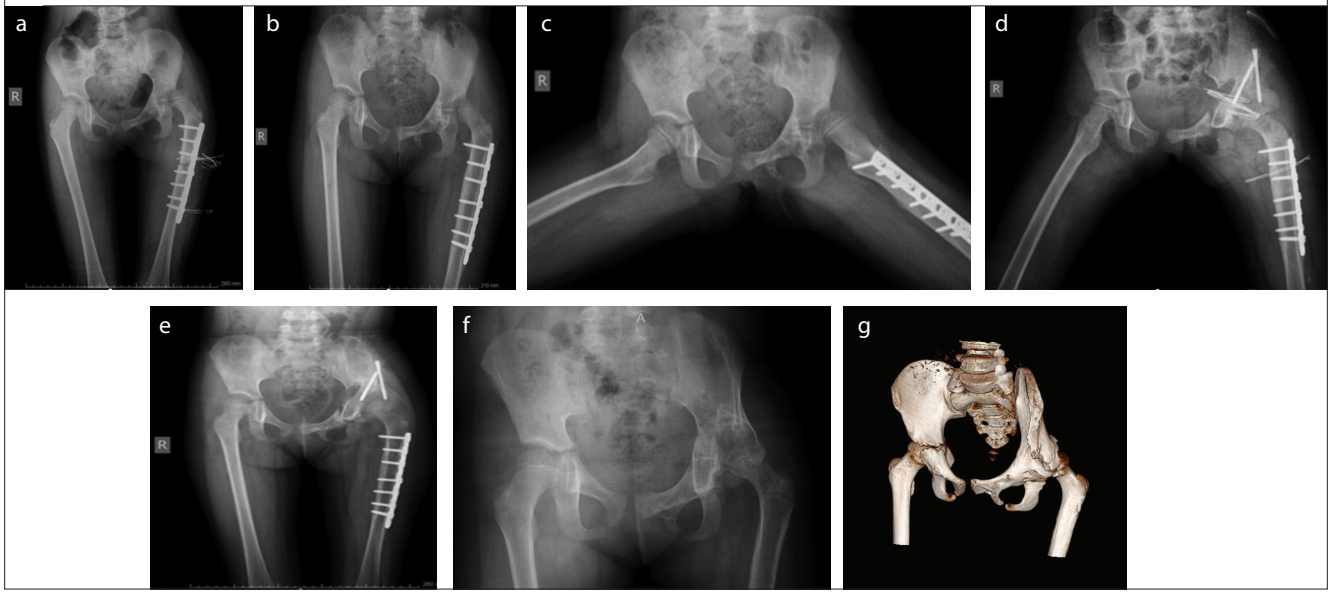


Figure 2. a-g. Postoperative plain radiographic images. (a) AP radiography of the left femur after correction of malunion (postoperative 1st day). (b) AP radiography of the left femur after correction of malunion (postoperative 6th month). (c) Lateral radiography of the left femur after correction of malunion (postoperative 6th month). (d) AP radiography of the left femur and pelvis after acetabular osteotomy (postoperative 1st day). (e) AP radiography of the left femur and pelvis after acetabular osteotomy (postoperative 3rd month). (f) AP radiography of the left femur and pelvis after acetabular osteotomy (postoperative 2nd year). (g) Three-dimensional pelvic computed tomography scan. (postoperative 2nd year)



As a first surgical step, we applied malunion osteotomy and plate and screw using an osteosynthesis technique for femoral problems using lateralization, Dynamic compression plate (DCP) and screw (Locking LC DCP Narrow Plate and 3.5 mm screw; Zimed, Gaziantep, Turkey) (Figures 2a-c). At 6 months postoperatively, we applied a triple innominate osteotomy to the acetabulum, thus achieving coverage of the femoral head (Figure 2d, e). The, postoperatively, (CE angle calculation was 12.6°. This operation utilized an ilioinguinal and posterior ischion incision technique. The patient exhibited decreased hobbling and stable CE angles as compared to initial postoperative calculations approximately 2-year postoperatively (Figures 2f, g).

These studies have been performed in accordance with the ethical standards laid down in the Declaration of Helsinki. For this purpose written patient consent form was obtained from the patient's parents to use this patient's information for a scientific study.

DISCUSSION

Research has determined that femoral fractures with same-sided triradiate injuries are rare (7). Infants are treated with bed rest, pelvic orthoses, skeleton traction, or a cast for pelvic fractures (4). Physicians choose conservative treatment because of its successful union and remodeling rates (3, 12). However, some studies

indicate that conservative treatment of displaced unstable pelvic fractures result in long-term morbidity and functional problems (6). McDonald (13) used conservative treatment on 15 infants diagnosed with unstable pelvic fractures and reported an increase in long-term morbidity, including delayed union, sacro-iliac fusion, and extremity length differences. Schwarz et al. (12) investigated anatomic reduction of pelvic ring fractures, indicating that conservative treatment may cause pelvic asymmetry. Our patient received conservative treatment for femoral and triradiate injuries; however, remodeling of the femur was insufficient and the acetabulum had ceased development as a result of acetabular dysplasia and iliac subluxation.

Bucholz suggests two simple patterns for triradiate trauma: a split-type as seen in Salter-Harris type-1/2 injuries; and crush-injury type as seen in type-5 traumas, which is hard to detect in X-ray imaging (14).We could not apply the Salter-Harris classification in this case because we could not get the first X-rays of the patient taken after the accident.

Furthermore, when we have consulted this patient, acetabular dysplasia and femoral head subluxation was already developed.

The patient's age is important for sequela formations in trauma. According to Bucholz, serious deformities have not been reported in traumas older than 11 years (14). In addition, according to Gebstein, when triradiate fusion is occurring acetabular dysplasia and hip dislocation with dysplasia occurs at half the normal rate and also, iliopubic fusion has a minimal effect on acetabular development (15). In our patient, all tree parts of TRC had closed prematurely, and the hip had also subluxated. Current literature suggested that almost half of the cases with TRC injuries cannot be diagnosed based on an analysis of radiography reports (8). As a result, patients diagnosed with femoral fractures should undergo examination of TRC and we recommend tomographic evaluations in suspicious cases to prevent further complications.

In cases exhibiting completely arrested development of TRC, studies have indicated good results following epiphysiodesis. Badina et al. (7) established that in all successful cases, epiphysiodesis had been applied before the development of femoral head subluxation. However, in our case, patient follow-up was poor, and clinical examination indicated hip subluxation. Thus, epiphysiodesis treatment was not considered. Trousdale and Ganz (5) indicated successful results with pelvic osteotomies in cases with a decreased coverage of the femoral head due to post-traumatic acetabular dysplasia and applied periacetabular osteotomy in two of the five cases exhibiting post-traumatic acetabular dysplasia, with good results. Scuderi and Bronson (9) applied a Chiari Osteotomy to one of the two cases with post-traumatic dysplasia with good results. The literature revealed no documentation concerning the application of acetabular triple innominate osteotomy to increase the femoral head coverage.

In our 8-year-old patient, triple innominate osteotomy increased the femoral coverage and balanced the CE angle to normal limits. After 2 year, the measurement was still 12.6°. In this study calculating the preoperative CE angle was not possible because of a subluxated hip. Hence, in our case CE angle measured postoperatively confirmed that triple innominate osteotomy may be the correct treatment.

CONCLUSION

We can state that triple osteotomy may also be considered as a treatment option in post-traumatic dysplasia cases when the femoral head covering is insufficient after our short time followed case experience. Furthermore, for patients exhibiting both hip dysplasia and malunion at the lower extremities, malunion should be treated prior to dysplasia. In addition, acetabular fractures of children can be missed in emergency services. For this reason, emergency physicians should be careful about its early diagnosis and complications.

Informed Consent: Written informed consent was obtained from the parents of the patient who participated in this case.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - G.B.S., M.C.C., A.B.; Design - G.B.S., M.C.C., A.B.; Supervision - G.B.S., A.B.; Resources - G.B.S., M.C.C., A.B.; Materials - M.D., A.B.; Data Collection and/or Processing - M.D., G.B.S., M.C.C.; Analysis and/or Interpretation - G.B.S., M.C.C., M.D.; Literature Search - G.B.S., M.C.C., A.B., M.D.; Writing Manuscript - G.B.S., M.D.; Critical Review - M.C.C., A.B., M.D.

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Case Report

A Rare Cause of Uptake of Radioactive Iodine by Non-Lactating Breast Tissue in A Patient with Papillary Thyroid Carcinoma: Prolactinoma

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ABSTRACT

We report a case of a post-menopausal woman who underwent total thyroidectomy due to papillary thyroid carcinoma followed by radioactive iodine therapy. After radioactive iodine therapy, total body scintigraphy (TBS) using radioactive iodine (I-131) was performed, and we observed uptake of I-131 by breast tissues. The scintigraphic and associated clinical characteristics of uptake of I-131 by breast tissues in a non-breastfeeding patient with thyroid cancer were unexpected findings. In this patient, elevated serum prolactin levels were documented at the time when the uptake of I-131 by the breast tissues was observed. We conducted hypophysis magnetic resonance imaging and detected a pituitary adenoma. Medical treatment with cabergoline was provided to this patient. When prolactin levels were normalized, uptake of I-131 by breast tissues was no longer evident on TBS.

Keywords: Papillar thyroid cancer, prolactinoma, uptake of radioactive iodine

INTRODUCTION

Total body scintigraphy (TBS) using radioactive iodine (I-131) has been a beneficial diagnostic procedure for evaluating the possible metastasis of differentiated thyroid cancer and the presence of residual thyroid bed remnants after surgery (1). Uptake of I-131 by normal non-lactating post-menopausal breast tissues is rarely observed. This finding may lead to misdiagnosis. It may appear to clinicians as a metastasis. We report a case of uptake of I-131 by the breast tissues in a patient with thyroid cancer who had hyperprolactinemia due to pituitary adenoma in the hypophysis.

CASE PRESENTATION

A 53-year-old female patient underwent total thyroidectomy and radioactive iodine ablation for papillary thyroid carcinoma (stage T2N0M0). Update of I-131 by breast tissues was observed on post-therapy TBS (Figure 1). She underwent mammography and breast sonography, and there was no lesion on the breast tissues. We found that the patient's prolactin level was 117 ng/mL (normal range, 5-20 ng/mL), but the patient had no symptom of hyperprolactinemia, such as galactorrhea. At this time, we found that the patient's thyroid stimulating hormone (TSH) level was 40 mU/L (normal range, 0-4 mU/L). We believed that the cause of the hyperprolactinemia was high the TSH level. We searched for other causes of hyperprolactinemia. But after TSH normalization with thyroid hormone replacement, we observed the persistence of prolactinemia. In euthyroid state, prolactin level was 143 ng/mL. There were no chronic diseases, such as chronic renal disease and liver cirrhosis. All laboratory studies returned to normal limits. She had not been using any drugs, especially antipsychotic drugs. When we investigated for hypothalamo-pituitary causes, we detected hypophysis adenoma via magnetic resonance imaging (Figure 2). We administered cabergoline (0.5 mg per week) to the patient. Her prolactin level improved to 12.6 mg/mL 3 months later. After prolactin level normalization, we performed TBS, and there were no update of I-131 by the breast tissues (Figure 3). Written informed consent was obtained from the patient.

DISCUSSION

The radioactive iodine treatment for differentiated thyroid carcinomas has its effect via sodium/iodide symporter (NIS). NIS is located in healthy and neoplastic thyroid cells and is stimulated by TSH (2, 3). This system has been shown in breast tissues during the lactation period and in some breast tumors. However, this system has not been shown in breast tissues that are not in the lactation period (4). The expression of NIS in breast tissues is under hormonal control. There are prolactin receptors on some breast tumors and in normal breast tissues during lactation (5). Therefore, recent

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Figure 1. Diffuse mild uptake by breast tissues was observed in scanned images after TSH normalization
TSH: thyroid stimulating hormone

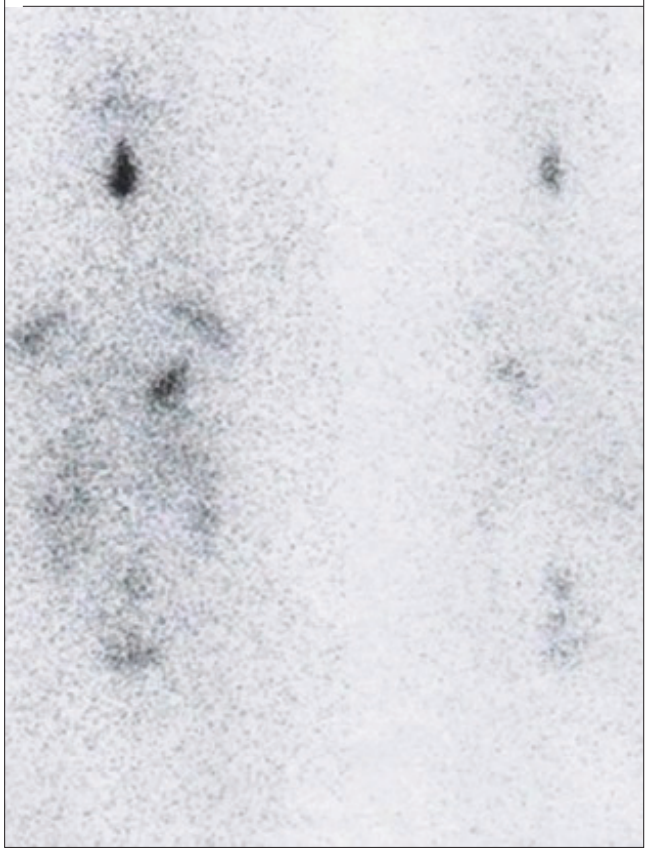


Figure 2. Typical MRI scan changes in pituitary adenoma. Coronal T1-weighted post-contrast MRI scan obtained for a 53-year-old woman who presented with pituitary adenoma and hyperprolactinemia
MRI: magnetic resonance imaging

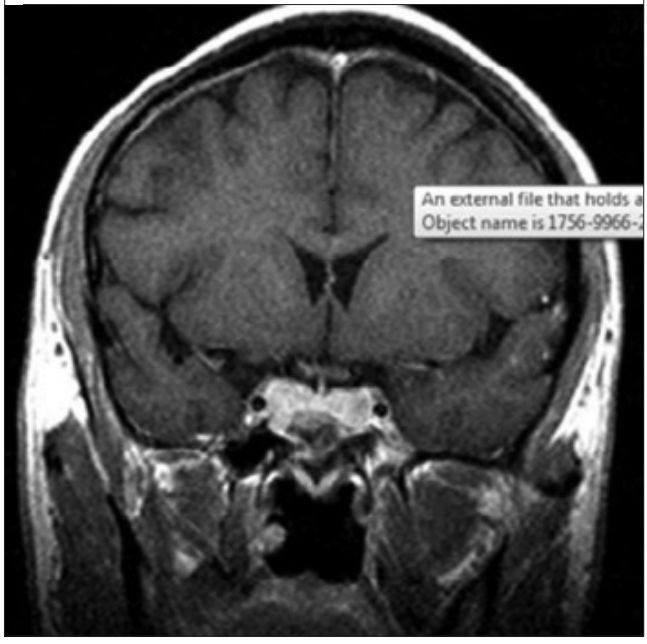
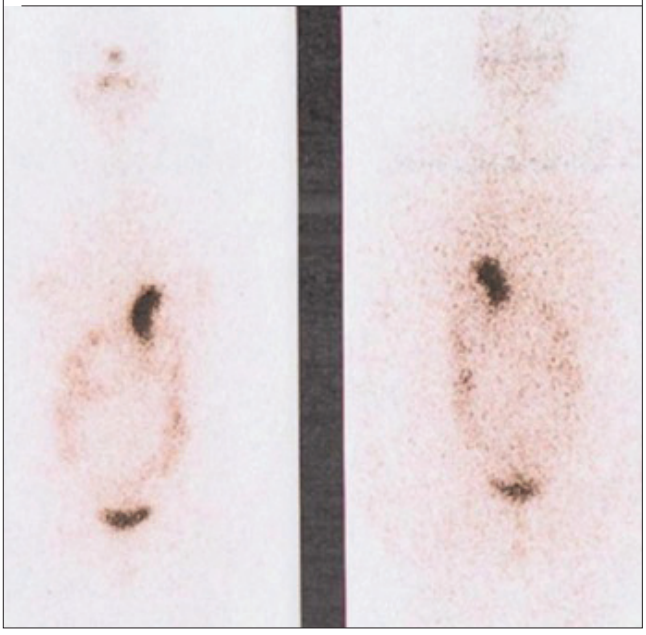


Figure 3. When prolactin and TSH levels were normalized (by use of dopamine receptor agonist), uptake by breast tissues was no longer evident on TBS
TSH: thyroid stimulating hormone; TBS: total body scintigraphy



experimental animal studies have shown that prolactin can stimulate he uptake of iodine by normal breast tissues (6). Bruno et al (7) administered radioactive iodine ablation to 302 patients with papillary and folicular thyroid carcinoma. Later, when they performed I-131 TBS, they found that, only 4 patients showed evidence of uptake of I-131 by breast tissues; in further examinations, prolactin secreting pituitary microadenoma was detected in only 1 patient. Further, 2 of these 4 patients had been exposed to a psychological chronic stimulation of prolactin and 1 to some unknown kind of agent responsible for the galactorrhea.

In another study, uptake of I-131 was observed in 2 patients with papillary thyroid carcinoma during TBS. But interestingly both patients were in post-menopausal period like in our case. Their breast tissue did not have lactation function (2). But both cases had specific features. One of the patient had been using risperidone that was an antidopaminergic drug (8). The other patient had chronic renal failure, and she was undergoing hemodialysis. In blood tests, both patients showed hyperprolactinemia. It is well known that chronic renal failure and risperidone can cause hyperprolactinemia (9, 10). They were treated with cabergoline, and their prolactin levels became normalized. After that, TBS was performed and no uptake of I-131 by breast tissues was observed.

Ahn et al. (11) reported a case of a patient with thyroid malignancy who underwent radioactive iodine therapy, and uptake of radioactive iodine by breast tissues was detected on post-treatment TBS. The patient had prolactinemia due to amisulpride.

CONCLUSION

This case reports the positive correlation between prolactin level and uptake of I-131 by breast tissues regardless of patients'

menopausal state or presence or absence of lactation. It makes us believe that endogenous hyperprolactinemia can be a reason for the significant uptake of I-131 by the tissues of non-lactating breasts, and hypophysis adenoma should be considered as a confounding factor.

Informed Consent: Written informed consent was obtained from patient who participated in this case.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - U.E., M.Ö., E.A.; Design - Z.A.S.; Supervision - U.E., M.Ö., E.A., Z.A.S.; Materials - U.E., M.Ö., E.A., Z.A.S.; Data Collection and/or Processing - Z.A.S., A.A.; Analysis and/or Interpretation - Z.A.S.; Literature Search - Z.A.S., A.A.; Writing Manuscript - Z.A.S., A.A.; Critical Review - U.E., M.Ö., E.A.

Conflict of Interest: The authors have no conflicts of interest to declare.

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Case Report

How Should Helicobacter Pylori Eradication Be Performed in Cases of Extensive Allergies to Proton Pump Inhibitors?

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ABSTRACT

In this presentation, we would like to discuss the path followed for drug selection in the case of a pediatric patient with extensive allergies to proton pump inhibitors (PPIs). A 15-year-old male patient presented with complaints of dyspepsia and epigastric pain for over a period of 4–5 years. It is known that there was a previous fixed drug eruption described with omeprazole and widespread rashes after lansoprazole. Famotidine treatment was initiated, but the patient was unable to use the drug because he presented with rash and itching 1 hour after drug intake. On physical examination, fixed drug eruption was observed in the whole body and gluteal region. Gastroduodenoscopy was performed. Macroscopically, the corpus and antrum were hyperemic, antrum and duodenum were nodular, and bulbus was normal. Multiple biopsies were taken. He was referred to the pediatric allergy department for evaluation of possible cross-reactivities between PPIs. In addition to skin prick and intradermal tests with famotidine and ranitidine, the patient underwent skin patch tests with all available PPIs. The pathologic result of biopsies was Helicobacter pylori (HP) (+++) with Giemsa staining. Because of the cross-sensitivity between PPIs and the positivity of the allergy tests, triple HP treatment was not considered. This is an interesting case because the patient had extensive allergies to all existing PPIs, and no similar cases have been reported yet in the literature. After evaluation of allergic tests, quadruple treatment without PPI (bismuth, ranitidine, metronidazole, and tetracycline) was initiated. HP treatment was assessed after 4 weeks, and two-step monoclonal stool HP antigen test was found to be negative.

Keywords: Eradication, extensive proton pump allergy, helicobacter pylori

INTRODUCTION

Helicobacter pylori (HP) eradication usually prevents ulcer recurrence and complications following appropriate proton pump inhibitor (PPI) treatment. HP eradication is also important in the treatment of stomach disorders such as MALT lymphoma, but it is controversial in gastric cancer prevention (1). Patients with penicillin allergies are provided with amoxicillin-free treatments. In this presentation, we would like to discuss the path followed for drug selection in a case of a pediatric patient with extensive allergies to PPIs.

CASE PRESENTATION

A 15-year-old male patient presented with complaints of dyspepsia and epigastric pain for over a period of 4–5 years. It is known that there was a fixed drug eruption described with omeprazole 3 years prior to presentation and widespread rashes in the body after lansoprazole in the previous year. Famotidine treatment was initiated, but the patient was unable to use the drug because he presented with rash and itching 1 hour after drug intake. On physical examination, fixed drug eruption was observed in his gluteal region (Figures 1a-d). One week later, gastroduodenoscopy was performed. Macroscopically, the corpus and antrum were hyperemic, antrum and duodenum were nodular, and bulbus was normal (Figures 2a-c). Multiple biopsies were taken. He was referred to the pediatric allergy department for evaluation of possible cross-reactivities between PPIs.

The patient underwent skin prick tests with famotidine and ranitidine (10 mg/mL and 40 mg/mL, respectively) and intradermal tests with famotidine and ranitidine (1/100 and 1/10 dilution). The patient also underwent skin patch tests with all available PPIs (10% and 30% concentrations). Skin patch test was found (++) positive with 30% concentration with lansoprazole and esomeprazole, whereas omeprazole was found to be negative (Figure 3). Omeprazole was not considered as a treatment option for the patient due to fixed drug eruption described with omeprazole 3 years prior to admission. Prick and intradermal tests were found to be negative with ranitidine. Oral provocation test with ranitidine was also negative. Prick and intradermal tests were

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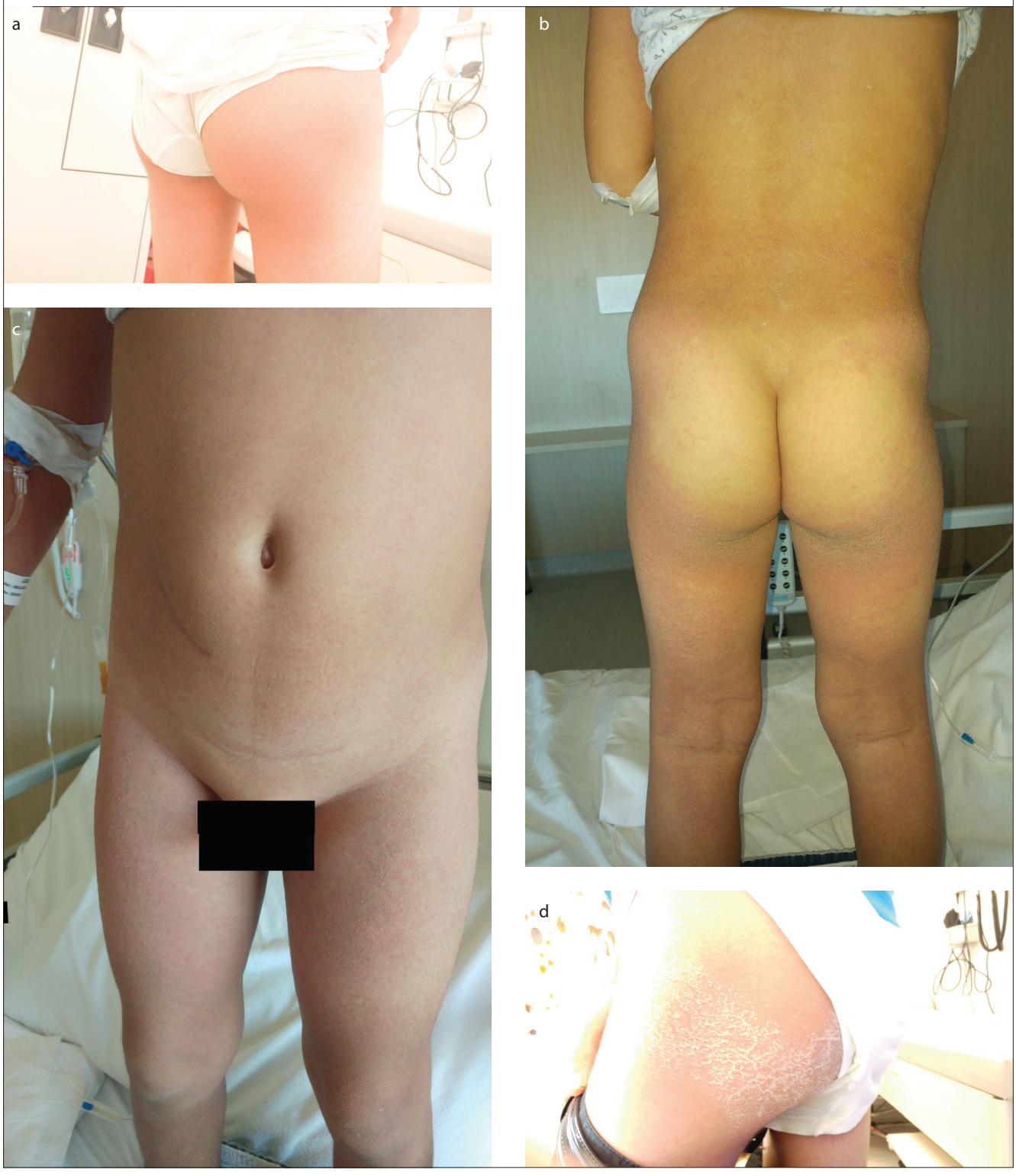
Figure 1. a-d. Fixed drug eruption described with famotidine (a-c). Healing period of fixed drug eruption and skin peeling (d)



negative with famotidine, but because of the recent reaction, it was not considered again.

The pathologic results of endoscopic biopsy was HP (+++) with Giemsa staining and there was no intestinal metaplasia. Because of the cross-sensitivity between PPIs and the positivity of the allergy tests, triple HP treatment was not considered. This is an interesting case because the patient had extensive allergies to all existing PPIs, and no similar cases have been reported yet in the literature. Quadruple treatment without PPI (bismuth, ranitidine,

Figure 2. a-c. Hyperemic and nodular antrum (a). Hyperemic and nodular antrum (b). Nodular appearance of duodenum (c)

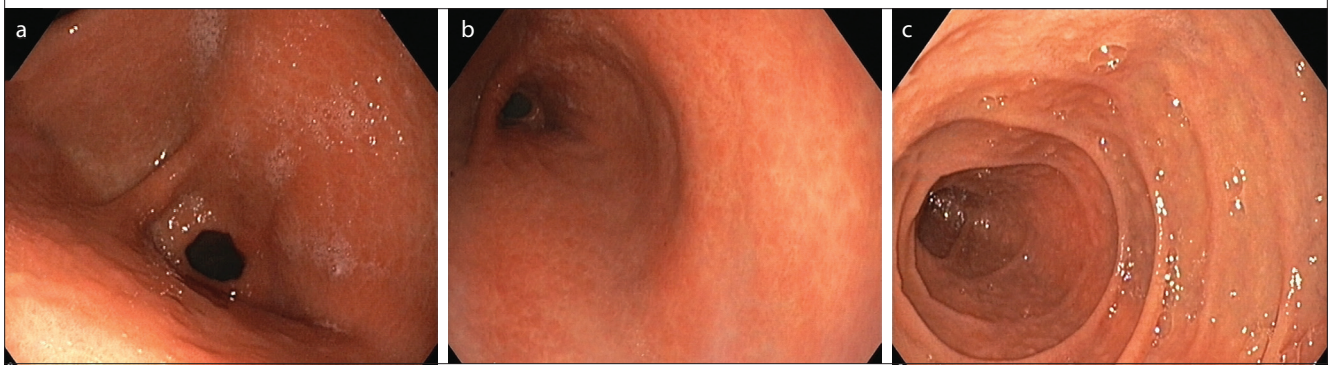
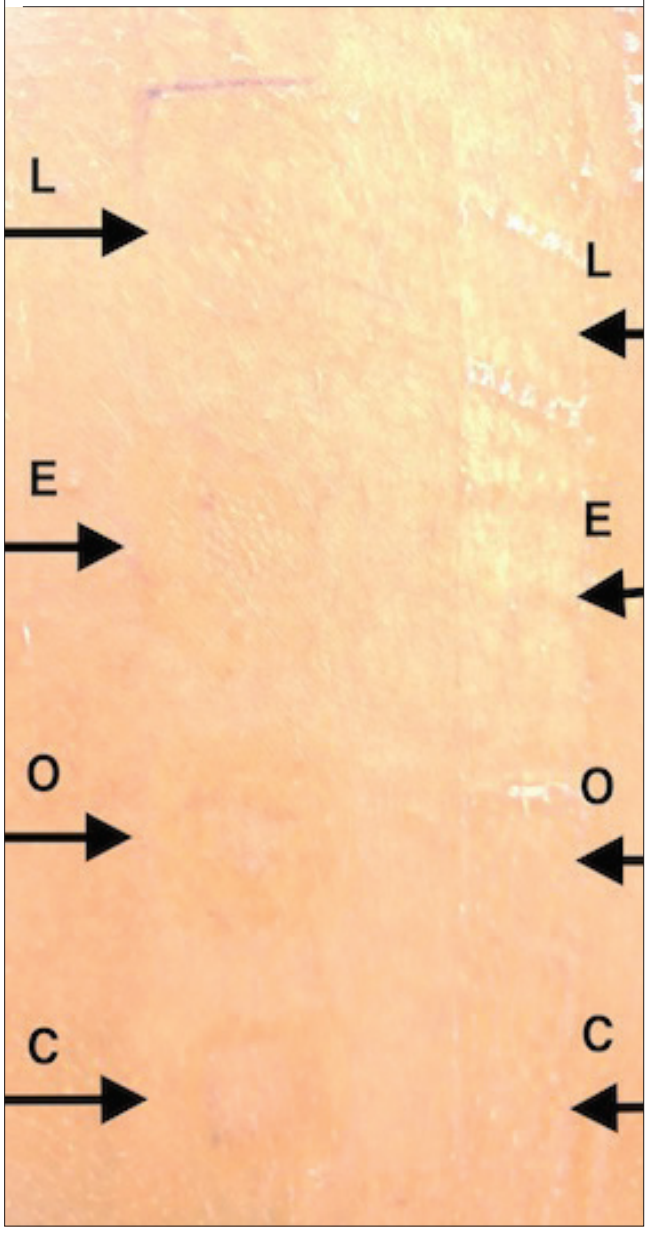


Figure 3. Skin patch tests with lansoprazole, esomeprazole, and omeprazole (right, 10% and left, 30% concentrations)
L: lansoprazole; E: esomeprazole; O: omeprazole; C: control with vaseline



metronidazole, and tetracycline) was initiated. He had no complaints after 2 weeks of this treatment. Following the completion of the treatment, HP treatment was assessed after 4 weeks, two-step monoclonal stool HP antigen test was found to be negative, and the patient was asymptomatic.

Written informed consent was obtained from the patient and his parents for the publication and presentation of case and images.

DISCUSSION

Despite the decreasing prevalence of HP infection, it still infects 30%–50% of the general population in western countries (2). HP causes active chronic gastritis in all infected persons and may cause complications, such as gastric malignancies, peptic ulcers, and dyspepsia. Its eradication usually prevents ulcer recurrence and complications (2).

The most frequently used initial treatment for HP is triple treatment including PPI, amoxicillin, and clarithromycin. If the patient comes from a place with increased resistance to clarithromycin, metronidazole is used instead (1).

All international guidelines agree that 14-day triple treatment with clarithromycin can be used as a first-line treatment for HP eradication (3).

If the strain is susceptible to clarithromycin and metronidazole in penicillin allergy, triple treatment with standard metronidazole should be provided instead of amoxicillin; if the strain is resistant to clarithromycin and the age of the patient is over 8, bismuth treatment with tetracycline should be provided instead of amoxicillin (4).

Helicobacter pylori eradication treatment is challenging because of developing resistance. Therefore, 2 or 3 antibiotics are usually given together with PPIs and/or bismuth-containing compounds for eradication (3).

Bismuth-containing quadruple treatment includes bismuth salt, tetracycline, and metronidazole in addition to a PPI (5). This treatment regimen has been previously proposed as the second-line treatment, since it is more complex than the standard treatment (6).

However, bismuth-containing quadruple therapy is a powerful weapon against antibiotic resistance as neither clarithromycin nor levofloxacin is involved. For this reason, bismuth-containing quadruple treatment has been returned in the last decade and is now also recommended as a first-line treatment (7).

International guidelines recommend using 2 standard doses of PPI to increase the effectiveness of antimicrobial drugs (3). This is because high intragastric pH reduces HP bacterial load and minimal inhibitor concentration of antibiotics (8).

With the increasing use of PPIs, significant treatment difficulties, side effects, and complications have occurred (6).

The case of this patient was interesting because of his extensive allergies to all available PPIs, and no similar case being reported yet in the literature. After evaluating the patient in terms of allergy tests, quadruple treatment without PPI (bismuth, ranitidine, metronidazole, and tetracycline) was provided to the patient to increase the effectiveness of the treatment. Instead of PPIs, we used ranitidine based on the results of the allergic tests. He had no complaints after 2 weeks of this treatment. HP antigen in stool was negative and he was asymptomatic after 1 month.

CONCLUSION

As a result, cross-reactivity between PPIs should be considered before HP treatment in patients with allergies to PPIs. The choice of treatment should be planned based on the results of allergic evaluations.

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