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Editöryal İletişim
Hacettepe Üniversitesi
Tıp Fakültesi Dekanlığı
06100 Sıhhiye - Ankara
E-posta: editor@actamedica.org

Yayıncı
Hacettepe Üniversitesi
Tıp Fakültesi Dekanlığı
06100 Sıhhiye - Ankara
Telefon: 0 312 305 10 80
Belgeç (faks): 0 312 310 05 80
E-posta: tipmaster@hacettepe.edu.tr

Yayıncılık Hizmetleri
Akdema Bilişim ve Yayıncılık
Telefon: 0 533 166 80 80
E-posta: bilgi@akdema.com
Web: www.akdema.com

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Ülkücan Kaplan

Editorial Office
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Hacettepe Medical School
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E-mail: editor@actamedica.org

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Hacettepe Medical School
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Phone: +90 312 305 10 80
Fax: 0 312 310 05 80
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REVIEW

A Review of Glutamate and Its Receptors: Their Roles in Brain Physiology and Pathology

Sevgi Uğur Mutluay[1]
ORCID: 0000-0002-8794-6397

Hülya Karataş[2]
ORCID: 0000-0003-4838-6320

[1]Ankara Yildirim Beyazit University, Institute of Health Sciences, Department of Translational Medicine, Ankara, Turkey.

[2]Hacettepe University, Institute of Neurological Sciences and Psychiatry, Ankara, Turkey.

Corresponding Author: Sevgi Uğur Mutluay
Ankara Yildirim Beyazit University, Institute of Health Sciences, Department of Translational Medicine, Ankara, Turkey.

E-mail: sevgimutluay@gmail.com

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ABSTRACT

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system. Through its ionotropic and metabotropic receptors it mediates both fast transmission and long term metabolic changes in a cell. Besides neurotransmission, it takes part in development of central nervous system, cell energy metabolism and synaptic plasticity processes. Glutamatergic signaling is strictly controlled. Under normal conditions, extrasynaptic gluatamate levels are maintained at low concentrations. Excessive transmission leads to excitotoxicity which results in cell damage and death. Glutamatergic dysfunction is involved in many pathologies including neuropsychiatric, neurodegenerative and neurodevelopmental disorders. Impairments in glutamate's physiological functions, excitotoxicity and disrupted modulation of other neurotranmitter systems contribute to these pathologies. This opinion aims to summarize the cellular mechanism that lead to pathology and review how these mechanisms translate into the clinic.

Keywords: Glutamate, NMDA, Excitotoxicity, Synaptic Plasticity, Pathology

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the central nervous system. Roughly, 80-90% of the synapses in brain are glutamatergic [1]. Glutamatergic synapses are tripartite synapses. Along with the pre- and postsynaptic neurons, astrocytes actively take a part in neurotransmission [2]. Glutamate receptors are divided into two main groups, as ionotropic and metabotropic [3]. Ionotropic glutamate receptors (iGluR) are ligand-gated ion channels, consisting of 4 subunits [1]. They mediate fast excitatory synaptic transmission [4]. They are further divided into three sub-groups that differ in pharmacological and electrophysicological properties [1]. These receptors are named after their selective agonists as; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) [5]. Each subunit of these receptor sub-groups may be encoded by distinct genes. In line with this, AMPA, KA and NMDA receptors may consist of combinations of different subunits as GluA1-4; GluK1-5; GluN1, 2A-D and 3A-B, respectively [1]. Subunit composition of receptors vary in different brain regions [3]. Morevover, in the course of physiologic (i.e. aging), and pathologic processes subunit expressions may change[6-8]. NMDA receptors are structurally more complex than AMPA and KA receptors. Alongside with the glutamate, there exist; binding domains for co-agonists, glycine and D-serine and allosteric modulators, Zn^{+2} and polyamines [9]. NMDARs are blocked by Mg^{+2} in a voltage-dependent manner and at resting membrane potentials the channel is in closed state [4]. NMDA receptors are permeable to Na^{+} and K^{+} as AMPA and KA receptors, however they are more permeable to Ca^{+2} than other sub-groups [1,9]. NMDAR activation requires; concurrent

binding of glutamate and glycine or D-serine, and removal of Mg^{+2} blockage by postsynaptic membran depolarization [1]. Metabotropic glutamate receptors (mGluR) are from family of G protein coupled receptors and coupled to downstream signaling cascades that act on much slower timescales [4]. There are 8 different mGlu receptors identified. Based on their aminoacid sequences, pharmacological characteristics and intra-cellular signaling pathways, these receptors are grouped into 3 classes [10]. Group I mGlu receptors (mGlu1, mGlu5) are coupled with Gq protein [1]. Ligand binding to these receptors leads to phospholipase C (PLC) stimulation, and subsequent activation intracellular signaling pathways which are mediated by inositol-3-phosphate (IP3) and diacylglycerol (DAG). Group II (mGlu2, mGlu3) and III mGlu (mGlu4, mGlu6, mGlu8, mGlu7) receptors are Gi protein coupled [1]. Activation of these receptors inhibits adenylate cyclase enzyme activity, and as a consequence cAMP mediated signaling pathways. Typically, Group I mGluRs are located postsynaptically, whereas Group II and III mGluRs are presynaptic, and also present on astrocytes [11]. Distribution patterns of these receptors also differ, such that, in spesific brain regions, certain sub-groups are expressed more frequently [10]. In the cell, glutamate is found to be concentrated in synaptic vesicles, and accumulation is mediated by vesicular glutamate transporters (VGLUTs) [1]. Normally, in relative to intracellular compartment, extracellular gluatamate levels are kept low, and this is maintained via synaptic and/or extrasynaptic excitatory aminoacid transporters (EAATs) [12]. EAATs remove glutamate from the extracellular space. In the mammals there are 5 type of EAATs identified. EAAT 1 and 2 are expressed on astrocytes and EAAT 3 on neurons [5].

Glutamatergic system takes part in maintaining the normal brain function, cognitive functions such as learning and memory, and also neurodevelopmental processes. Accordingly, glutamatergic system dysregulation is involved in many psychiatric and neurodegenerative diseases. In the first part of this review, cellular mechanisms of physicologic and pathologic events where glutamatergic system plays a role will be outlined, then their manifestations in clinic will be discussed.

Cellular mechanisms

Cellular Energy Metabolism

In the neurons, glutamate is synthesized from α-ketoglutarate by addition of an amino group to the molecule. α-ketoglutarate is one of the intermediates formed during Krebs cycle. Glutamate in the synaptic cleft is taken up by glia. Here, in the glia, glutamate reacts with free ammonia, resulting in glutamine synthesis. Glutamine is released from glia to extracellular space and taken up by neurons. In the neurons, glutamine is converted back to glutamate by glutaminase. This metabolic pathway between neurons and glia is called glutamate-glutamine cycle. Glutamate-glutamine conversion in the neurons is of importance with regard to cellular energy metabolism. Some part of newly formed glutamate is converted to α-ketoglutarate by glutamate dehydrogenase. In this way Krebs cycle and energy production are sustained [1]. Moreover, it was suggested that; glutamate presence in astrocytes causes a shift in energy production mechanisms from oxidative metobolism to glycolysis, and then, lactate -the end-product of glycolysis- is used by neurons as a substrate for oxidative phosphorylation [13]. Glutamate may also play an important role in nitrogen homeostasis. There is evidence that glutamate acts as a nitrogen buffer during α-ketoglutarate and glutamine conversions, and this is applicable for both normal and hyperammonemic conditions [14].

Neurogenesis

Neuronal cells, astrocytes and oligodendrocytces originate from embryonic progenitor cells. Progenitor cells continue to exist in some regions of postnatal and adult brain and found to be involved in neurogenesis during these periods [15]. It was reported that glutamate receptors expressed on progenitor cells; moreover proliferation, migration and differentiaton of the cells are modulated by glutamatergic system. In line with this, AMPA/KA, NMDA and mGlu5 receptors were shown to mediate neurogenesis during embryonic stage, after birth and adulthood [15-17].

A recent study investigated the glutamate receptors in various regions of Wistar rat brains of different age groups by using quantitative *in vitro* receptor autoradiography [18]. AMPA receptor

densities were significantly higher ($p < 0.01$) in P90 rats compared to P0 in all brain regions investigated (olfactory bulb: 262%; striatum: 311%; hippocampus: 321% and cerebellum: 471%). Although the course of changes between age groups was comparable in all examined areas, their peak varied for the different brain regions. Between P0 and P10, a significant increase was found in the olfactory bulb, the striatum and the hippocampus, but not in the cerebellum. Significant changes were found between P10 and P20 only in the cerebellum and between P20 and P30 in the striatum and cerebellum. KA receptor densities were significantly higher in P90 rats compared to P0 in all brain regions investigated including olfactory bulb, striatum, hippocampus and cerebellum. Between P0 and P10, densities increased significantly in the olfactory bulb, striatum and hippocampus. Furthermore, between P10 and P20, a significant increase was found only in the striatum and cerebellum, whereas no significant changes were found between P20 and P30. Interestingly, between P30 and P90, there was a decrease in receptor densities, though it only reached significance in the striatum. NMDA receptor densities were below the detection limit in the brain of P0 rats and in the cerebellum at all ages. In the olfactory bulb, NMDA receptor densities were higher at P90 than P10, whereas the opposite was true for the striatum and hippocampus. Between P10 and P20, NMDA receptor densities increased significantly in the hippocampus. No significant changes were found between P20 and P30. Between P30 and P90, a significant decrease was found in the striatum and hippocampus [18]. The analysis of mRNA expression levels of NMDA receptor subunits revealed NR2B expression predominately in the first postnatal week, and NR2A expression in the following weeks [19-21]. This subunit switch is thought to play a developmental role in NMDA neurotoxicity [22] and indicates the importance of neonatal neurotransmission mediated by the NMDA receptor[19]. During synaptogenesis, there is an increase in the numbers of synapses until reaching its peak after P28, but the numbers then decline slowly during maturation, a process known as synapse elimination [23,24]. Hence, the decline of receptor densities in striatum and hippocampus from P30 to P90 may be due to synapse elimination to redefine or rather fine-tune neuronal networks. Moreover, NMDA receptor activation results in the insertion of AMPA receptors and alters dendritic spine morphology, which is important for the stability and maturation of synapses [25].

Modulation of Synaptic Transmission

mGlu receptors change activity of voltage and ligand gated ion channels via intracellular signaling pathways of which they are coupled with. Each of three sub-groups inhibits L-type voltage-gated Ca^{+2} channels, group I and II receptors also inhibit N-type voltage-gated Ca^{+2} channels [1]. Besides, they alter excitability of the cell by regulating the activity of various K^{+} channels [9]. On the postsyanptic neurons, activation of mGluR, modulates ligand-gated receptors such as; NMDA, KA, GABAA. The context of this modulation may vary and may be ion channel activation or inhibition in different tissues [1]. Group II and III mGluRs on the presynaptic neurons function as autoreceptors. It has been shown that their activation inhibits neurotransmitter release from glutamatergic, GABAergic and monoaminergic neurons [26].

Synaptic Plasticity

Synaptic plasticity, very broadly, can be defined as strengthening and/or weakening of synaptic connections between neurons. In terms of functionality, it primarily serves to learning and memory processes. Moreover, it has been shown that it plays a crucial role in development of neural circuits. It has been observed in many brain regions and there are multiple types of it. Long term potentiation (LTP) and long term depression (LTD) are two types of synaptic plasticity, of which studied the most [27].

At the molecular and cellular level, it was observed that LTP occurs via different mechanisms in distinct circuits of the same tissue [28]. LTP in Schaffer collaterals of the hippocampus is mediated by NMDA and AMPA receptors.

During the induction phase of LTP, a sequence of events occurs as follows; postsynaptic AMPAR activation, postsynaptic neuron depolarization, removal of Mg^{+2} blockage from NMDAR, increased Ca^{+2} influx into the postsynaptic neuron and finally activation of Ca^{+2} dependent kinases. Next, AMPARs in the synaptic pool are inserted to the membrane, meanwhile, protein kinase C induction increases

AMPAR permeability. Collectively, increased number of AMPARs and their permeability enhance synaptic transmission efficacy.

It was shown that, NMDA and AMPA receptors in Schaffer collaterals also features in LTD [28]. In this form of synaptic plasticity, unlike in LTP, synaptic efficacy is decreased. This is achieved by stimulating postsynaptic neurons at low frequencies for extended time periods. As a result of low frequency stimulation, postsynaptic neuron is less depolarized and Ca^{+2} influx to the neuron is lower when compared to LTP activity [27]. Ca^{+2} at low concentrations activates calcium-dependent phosphatase calcineurin, which has a highger affinity to Ca^{+2} than CaMKII. Calcineurin dephosphorlyates and phosphorlyates AMPAR subunits, GluR1 and GluR2, respectively, which results in AMPAR endocytosis [28].

Besides iGluRs, mGluRs are also involved in LTD. Underlying mechanisms of mGluR mediated LTD is not clear yet. Still, there are several pathways and gene transcription products which were proposed as mediators of AMPAR internalization [29]. In this context, MAPK and mTOR pathways are especially thought to be important for mGluR mediated synaptic plasticity [26].

It is likely that, apart from hippocampus, glutamatergic sytem participates in synaptic plasticity in basal ganglia as well. It was observed that NMDAR activity here alters LTP, LTD and striatal learning performance by modulating the dopaminergic system [30,31].

Excitotoxicity

Excessive glutamatergic transmission disrupts normal cellular functions by triggering a set of events in the cell. These events are often interrelated, they cause neural damage and death, and the process is called excitotoxicity. Excitotoxicty usually occurs when extracellular glutamate levels are high. However, in hypoxic and hypoglycemic conditions, where cellular homeostasis is impaired, even non-toxic glutamate levels are detrimental [32]. In most of the excitotoxic events, Ca^{+2} is involved. Extracellular glutamate increases intracellular Ca^{+2} by binding to AMPA, NMDA and group I mGlu receptors. In the cell, Ca^{+2} activates several enzymes such as proteases, phospholipases and endonucleases [33]. These enzymes induce multiple events including; mitochondrial damage, lipid peroxidation, increase in reactive oxygen species, DNA damage, endoplasmic reticulum dysfunction and acidosis [9]. As a result, cell undergo apoptosis and/or necrosis. Pathological conditions also cause glutamate transporter dysfunctioning. Impaired re-uptake of extrasynaptic/nonsynaptic glutamate to the cells and/or glia is another factor that contributes to excitotoxicty [9,32].

Clinical Pathologies

Migraine

There is increasing evidence indicating a role for glutamate in migraine. Levels of glutamate are higher in the brain and possibly also in the peripheral circulation in migraine patients, particularly during attacks [34]. Population based genetic studies point genes that are involved with glutamate signaling in migraine, and gene mutations responsible for familial hemiplegic migraine and other familial migraine syndromes may influence glutamate signaling. Animal studies indicate that glutamate plays a key role in pain transmission, central sensitization, and cortical spreading depression. Multiple therapies that target glutamate receptors including magnesium, topiramate, memantine, and ketamine have been reported to have efficacy in the treatment of migraine [34].

A recent study shows the first direct support of a threshold level of extracellular glutamate for spreading depolarization (SD), that is electrophysiological corralate of migraine aura, ignition regardless of genotype [35]. This study defines a new glutamatergic release mechanism which is called glutamatergic plumes. Plumes are non-canonical, calcium-dependent glutamate signaling events, driven by both astrocytic glutamate clearance impairment and neuronal action-potential-independent release that occur spontaneously in the FHM2 model of migraine with aura and in wild type mouse brain [35]. The prediction of the model is that increased susceptibility to SD in both FHM1 and FHM2 [36,37] is due to the fact that the glutamate threshold is reached with stimuli of lower intensity. Given that impaired glutamate clearance primarily affects the activation of NMDA receptors [38,39]. This study suggests a possible role of plumes in the cooperative activation of NMDA receptors necessary for SD ignition [35].

The association of plumes with SD and with experimental conditions relevant to neuronal injury proposes that plumes may represent a broadly relevant mechanism of neurological disease.

Epilepsy

Epilepsy is a chronic disease characterized by spontaneous and recurrent seizures. Epileptic seizures result from overstimulation and synchronized discharge of neurons. Spontaneous and recurrent seizures develop gradually over time as neuronal networks become more excitable, and this process is called epileptogenesis [40].

Epileptic seizures represent increased glutamatergic and decreased GABAergic transmission. NMDAR and AMPAR agonists induce epileptic seizures both in animals and humans [41]. Besides, in patients with epilepsy, elevated glutamate levels during post-ictal phase have been shown *in vivo* in many studies [5]. Excess glutamate in extracellular compartment, which enhances synaptic efficacy, is viewed as a contributor to epileptogenesis. On that note, activation of AMPAR and NMDAR, that are both involved synaptic plasticity, becomes prominent. Mutations in NMDARs and, to a lesser extent in AMPARs, were reported to be linked to epilepsy [41]. In epilepsy models, it was observed that number of NMDARs was increased, presynaptic mGlu2/3 receptors was decreased and GABARs were internalized following *status epilepticus* [7]. As a result of these events, which enchance glutamatergic signaling and release, it is likely that cells becomes more excitable. Within this context, glutamate re-uptake mechanisms are also under consideration. It was reported that number of glial transporters declined in both patients and animal models of the disease [7]. Another part for glia in the pathology, may be related to their role in glutamate and cell energy metabolism. It was proposed that, astrocytic dysfunction leads to increse in extracellular glutamate levels and cell excitability, as a result of disrupted glutamate-glutamine cycle [42].

Antiepileptic drugs used today act on glutamatergic system either directly or indirectly. However, 30% percent of the cases is resistant to treatment. With agents that modulate neuron-glia interaction and cell energy metabolism, it seems possible to make progress within this treatment-resistant population.

Alzheimer's Disease

The pathophysiology of Alzheimer's disease (AD) is highly complex. Several processes such as; formation amyloid ß (Aß) peptide and Tau protein, mitochondrial damage, oxidative stress, inflammation, cholinergic dysfunction are known to be involved in the pathology. Among others, Aß peptide formation is one of the main contributors to the disease development, and found to cause glutamatergic system dysfunction. Previous stuides report that Aß peptides disrupt glial EAAT2 functioning [12], alter the expression of presynaptic proteins which take part in neurotransmitter release, and directly enhance NMDAR mediated synaptic transmission and elevate D-serine levels [31]. It was also proposed that, in AD, astrocytic glutamate levels and extrasynaptic NMDAR activity are increased [31,43]. Available data supports the idea that increased glutamatergic signaling mediate excitotoxicty and neurodegeneration in AD [3].

Parkinson's Disease

In Parkinson's disease, primary pathology is degeneration of nigrastriatal dopaminergic neurons and subsequent depletion of striatal dopamine. Secondary to the dopaminergic deficit, glutamatergic dysfunction is thought to be implicated in motor impairments and progressive neurodegeneration [44,45]. Results from animal studies indicate that, in the basal ganglia, subunit compositon and expression of iGlu and mGlu receptors are altered [8]. Moreover, gluatamatergic activity is increased [10]. When combined with dopaminergic agents, NMDAR and AMPAR antogonists have been found to be beneficial to improve motor functions and Levodopa induced dyskinesias (LIDs)[8]. NMDAR antagonist amantadine is currently in clinical use for LIDs. It was also suggested that mGluR modulation might be an effective treatment modality for LIDs, as well as provide neuroprotection [8,46]. Within this scope, several agents from group I mGluR antagonists, negative allosteric modulators, group II and III agonists, positive allosteric modulators have been tested preclinically and results have been promising [10].

Huntington's Disease

Huntington's disease (HD) is a genetic disorder that is mostly inherited. It manifests with motor

impairments -chorea being the most dominant- and accompanying psychiatric and cognitive symptoms [10]. In HD, key pathalogical finding is degeneration of cortical neurons and basal ganglia. Dopaminergic and glutamatergic imbalance is thought to be implicated in chorea [47]. It is also likely that, as in other neurodegenerative diseases, glutamate excitotoxicity elicit neuronal death. It was shown both in animal models and HD patients that glutamate transporter GLT-1/EAAT2 expression is reduced [48]. In line with this, agents that decrease glutamate release via mGluR2/3 activation are suggested to be as candidate drugs for treatment [10].

Ischemic Stroke

Glutamatergic system is directly related to neuronal death after stroke. In ischemic tissues, energy homeostasis is disrupted. Hypoxic conditions cause activation of voltage gated Ca^{+2} channels on the presynaptic neuron, and reverse sodium-calcium exchange both in neurons and astrocytes [3,49]. Increased Ca^{+2} in the cell triggers glutamate release. It was also reported that; under ischemic conditions, neuronal and glial glutamate transporters operate in the reverse mode and/or their expression levels are reduced [5,49]. For the treatment of stroke; NMDAR antagonists, which reduce increased glutamatergic signaling and prevent subsequent excitotoxicity, were found to be successful in animal studies, but failed in clinical trials [50]. However, by targeting the intracellular processes which follow NMDAR activation and provoke cell death, it seems possible to obtain more favorable results for the treatment.

Neurodevelopmental Disorders

Neurodevelopmental disorders are a cluster of conditions that include autism, attention deficit/ hyperactivity disorder, Down syndrome, Rett syndrome, Fragile X syndrome and mental retardation. They are primarily related developmental anamolies of central nervous system and characterized by motor, cognitive and emotional symptoms.

In the recent studies, it was reported that disrupted formation of glutamatergic synapses might have a role in neurodevelopmental impairments, hence glutamatergic dysfunction was suggested as a common underlying mechanism in these disorders. Mutations were shown in genes encoding AMPA, NMDA, mGlu receptors and postsynaptic density proteins [51]. Biochemical and post-mortem analysis revealed elevated glutamate levels in patients with autism [52,53]. The reason for this increase was suggested to be disrupted glutamate/ glutamine metabolism as a result of gliolisis and decline in glutamate decarboxylase enzyme [52]. Further, in clinical trials, autism symptoms improved with glutamate antogonists memantine and amantadine [53].

Similarly to autism, glial cells are thought to be implicated in attention deficit/hyperactivity disorder (ADHD). It was shown that, neuroinflammatory responses that was controlled by glial cells and also extracellular glutamate levels were altered in ADHD [54]. Modulatory effect of glutamatergic system on dopaminergic neurons is a proposed mechanism as a contributor to ADHD pathology [55].

Depression

Major depression is a serious health condition, and is quite common on a global scale. According to latest WHO data, it affects more than 260 million people worldwide [56].

Known mechanisms underlying depression are varios and rather complex. As well as neurochemical and immunological impairments, genetic susceptibility end enviromental factors are major contributors to the etiology [57]. Monoamine hypothesis, that was proposed in 1950s, predicts an imbalance between serotonin, noradrenaline and dopamine levels in the brain [58]. Many medications used today are developed on the basis of this theory. Even so, there are limitations to monoamine hypothesis and it does not account for the disease pathophysiology alone [59].

In early 2000s, upon the observations that NMDAR antagonist ketamine has acute antidepressant effects, the connection between glutamatergic system and depression became a popular topic for researchers. In the studies conducted on patients with depression; serum/plasma glutamate and cerebrospinal fluid glutamine levels were reported to be increased [5,58], gluatamate in prefrontal cortex and anterior cingulate cortex were shown to be decreased[60]. Glial reduction was also observed and this was suggested to be related to glutamate excitotoxicty [5,58]. Moreover, in post mortem

studies, variations have been reported in NMDA and AMPA subunit expressions for individuals with unipolar and bipolar disorders [5,11,60]. These data indicate a strong relationship between depression and glutamatergic dysfunction. Besides, elucidation of ketamine's mechanism of action in depression, contributed to a better understanding of the disease pathophysiology at the molecular level. Ketamine antagonizes NMDARs on GABAergic interneuros, thus increases glutamate release. Subsequent to gluamate increase, intracellular Ca^{+2} are elevated by postsynaptic NMDARs and AMPARs activation, and BDNF release is triggered [58,59]. BDNF enhances mTORC1 signaling via ERK-AKT pathway. mTOR pathway activation induces intracellular protein synthesis such as BDNF, GluA1 and PSD95 [11]. In addition, it was reported that ketamine blocks extrasynaptic NMDA receptors on the postsynaptic cell. Net effect of this action is disinhibition of eEF2 and increased synthesis of BDNF and synaptic proteins [11]. These data support the involvement of synaptic plasticity and glutamatergic system in depression. In line with the ketamine's effects on NMDAR and AMPAR, there are several NMDAR antagonists and AMPAR positive allosteric modulators in development as candidate therapeutics. Less data are available in regard to metabotropic receptors, however it was shown in preclinical studies that mGluR2/3 and mGluR5 antogonists possess ketamine-like effects [61,62].

Anxiety Disorders

Anxiety disorders are often co-occur with depression. In the treatment, serotonine reuptake inhibitors and benzodiazapeines are commonly used. Current treatment approaches are successful ameloriating the symptoms, still there is a need for superior treatments in terms of efficacy and side effect profile [63]. Neural substrate for the stress response is corticolimbic circuits, that includes GABAergic, dopaminergic and serotoninergic systems. It is known that, being the major exciatatory neurotransmitter, glutamate modulates these systems.

Since depression and anxiety are concomitant diseases, and known underlying mechanisms overlap to some degree, it is possible that glutamatergic system is also involved in anxiety. In precilinical studies, it was observed that; acute stress increases glutamate in prefrontal cortex and limbic structures, whereas chronic stress downregulates glutamate receptors and decreases transmission efficacy [64]. Data from human imaging studies are inconsistent. Still it was reported that gluatamate levels in prefrontal cortex were elevated [64], and gluatamate/creatinine ratio in anterior cingulate cortex correlated with anxiety scores [5]. Within this context, agents that target glutamatergic system and act via different mechanisms were tested in animal and human studies. NMDAR antagonists ketamine and memantine, NMDA partial agonist D-cycloserine, glial cystine-gluatamate transporter modulator N-acetylcysteine, voltage gated Ca^{+2} channel blocker riluzole are some of them [63,64]. In these studies, drugs that decreases glutamatergic transmission showed anxiolytic effects. Yet, these were open label clinical studies conducted with small-size populations. For the use of glutamatergic agents as therapeutics, evidence from larger population-based randomized controlled studies is needed.

Obssessive compulsive disorder (OCD) is aetiologically known to be linked to dysfunction of cortico-striatal-thalamic circuit. Studies indicate that glutamatergic signaling might be involved in dysfunctioning of these pathways [5,63,64]. It is not clear yet how different glutamate receptors are implicated in the disease pathophysiology. In clinical trials glutamate antagonists; memantine, ketamine, topiramate and glutamate release inhibitors such as riluzole and lamotrigine have been tested [65]. But, as in anxiety studies, results obtained are not consistent and more studies with larger sample sizes should be conducted.

Schizophrenia

Schizophrenia is a serious psychiatric disorder, characterized by positive (hallucinations, delusions, disorganized thinking), negative (affective impairment, anhedonia, antisocial behaviour) and cognitive (attention deficit, learning and memory impairments) symptoms [66]. It is a multifaceted illness that has genetic, enviromental and prenatal risk factors.

The very first theory relating to underlying disease mechanism is dopamine hypothesis. It states that, in schizophrenia, activity of mesolimbic dopaminergic pathway is increased [67]. The hyptohesis is proved to be true many times with pre-clinical and clinical data, however it falls short on explaining some other aspects of the disease. In line with the latest

available data, it is now known that, serotonine and glutamate play a role in disease pathophysiology. Glutamatergic dysfunction is considered to be a better model for explaining the negative and cognitive symptoms [68]. Moreover, it was suggested that all three systems, dopaminergic, serotoninergic and glutamatergic, are linked to psychosis [69].

The glutamate hypothesis of schizophrenia emerges from the discovery that NMDAR antagonists phencyclidine and ketamine cause schizophrenia-like symptoms [70]. According to this theory, in schizophrenia, NMDARs on cortical GABAergic interneurons are hypofunctional. Net effect of this hypofunctionality is disinhibiton of interneurons, elevated glutamate levels and increased glutamatergic transmission [68]. Glutamatergic dysfunction was shown in animal studies and in humans with *in vivo* imaging techniques [66,68,70]. In genetic studies, risk allells linked to certain GluR subunits have been identified, and also variations in receptor subunit expression have been reported in post-mortem studies [66]. In clinical studies, several drugs that modulate NMDAR activity or glutamate release have been tested, but none of them found to be effective as much as dopamine receptor blockers [70].

It is worth noting that there is an extensive interaction between dopaminergic and glutamatergic systems. Thus, for the design of future studies, it seems as a better approach to consider the relationship between glutamate and dopamine.

CONCLUSION

Glutamate is a unique neurotransmitter in terms of its functionality. It excites almost every neuron in the brain. Along with signal transmission, it has a part in many key processes such as; central nervous system development during prenatal period, maintanance of cell homeostasis and synaptic plasticity. Glutamatergic signaling is essential to sustain vital functions, however excess signaling results in cell death. Since the glutamate is found widespread and features in critical events in the brain, glutamatergic system dysregulation contributes to pathophysiology of several diseases. Known mechanisms of neuropsychiatric, neurodegenerative and neurodevelopmental disorders are complex and multifactorial, still, in all cases, signal transduction and cell energy metabolism are disrupted. Synaptic plasticity and excitotoxicity are more prominent for some of these diseases, and these topics could be of great interest for further research.

Abbreviations

Aß	Amyloid Beta
AD	Alzheimer's Disease
ADHD	Attention Deficit/Hyperactivity Disorder
AKT	v-Akt Murine Thymoma Viral Oncogene
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA Receptor
BDNF	Brain Derived Neutrophic Factor
cAMP	Cyclic AMP
DAG	Diacylglycerol
EAAT	Extrasynaptic Excitatory Aminoacid Transporter
eEF2	Eukaryotic Elongation Factor 2
ERK	Extracellular-regulated Kinase
FHM1	Familial Hemiplegic Migraine 1
FHM2	Familial Hemiplegic Migraine 2
GLT	Glutamate Transporter
GABA	Gama Amino Butiric Acid
HD	Huntington's Disease
iGluR	Ionotropic Glutamate Receptor
IP3	Inositol-3-phosphate
KA	Kainate
LID	Levodopa Induced Dyskinesia
LTD	Long Term Depression
LTP	Long Term Potentiation
MAPK	Mitogen Activated Protein Kinase
mGluR	Metabotropic Glutamate Receptors
mTOR	Mammalian Target of Rapamaycin
mTORC1	Mammalian Target of Rapamycin Complex 1
NMDA	N-methyl-D-aspartate
NMDAR	NMDA Receptor
PD	Parkinson's Disease
PLC	Phospholipase C
PSD95	Postsynaptic Density Protein 95
SD	Spreading Depolarization
OCD	Obssessive Compulsive Disorder
VGLUT	Vesicular Glutamate Transporter
WHO	World Health Organization

Author contribution

Conception and design: SUM and HK; literature review: SUM and HK; draft manuscript preparation: SUM and HK. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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acta medica

ORIGINAL ARTICLE

Monitoring Serum Lipid Profile and Liver Transaminase Levels During Isotretinoin Therapy

Duygu Gülseren[1]
ORCID: 0000-0003-1602-726X

Ecem Bostan[1]
ORCID: 0000-0002-8296-4836

Neslihan Akdoğan[1]
ORCID: 0000-0002-1137-5399

Başak Yalıcı-Armağan[1]
ORCID: 0000-0001-9745-1331

Sibel Doğan[1]
ORCID: 0000-0002-5383-6886

Sibel Ersoy-Evans[1]
ORCID: 0000-0002-5040-2476

Gonca Elçin[1]
ORCID: 0000-0001-6292-7272

Ayşen Karaduman[1]
ORCID: 0000-0002-4056-6303

[1]Hacettepe University, School of Medicine, Department of Dermatology, Ankara, Turkey.

Corresponding Author: Duygu Gülseren
Hacettepe University, School of Medicine, Department of Dermatology, Ankara, Turkey.
E-mail: duygu_gulsrn@hotmail.com

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ABSTRACT

Objective: Isotretinoin is generally chosen as the first line treatment of nodulocystic acne. Close laboratory monitoring is a general practice for many dermatologists to be on the safe side due to the laboratory side effects of isotretinoin. We aimed to determine the laboratory side effects of oral isotretinoin and optimal frequency interval for laboratory monitoring during isotretinoin treatment.

Materials and Methods: One hundred patients who were under oral isotretinoin therapy were included in the study; serum lipid levels along with liver transaminase levels were recorded at the baseline, 1-, 3- and 6- months of the therapy.

Results: We found that there might be slight elevations in serum aspartate transaminase and lipid levels during isotretinoin therapy ($p<0.05$, for all). However, statistically significant elevations were observed within the first month of isotretinoin therapy.

Conclusions: Frequent laboratory monitoring might not be necessary for all acne patients undergoing isotretinoin therapy. Patients should be screened at the first month of the therapy and then, the intervals can be extended.

Keywords: Acne vulgaris, isotretinoin, medical therapy

INTRODUCTION

Oral isotretinoin has been widely used by dermatologists since its approval by the US Food and Drug Administration (FDA) for the treatment of severe nodulocystic acne in 1982 [1]. However, many dermatologists do not feel comfortable when prescribing isotretinoin due to its clinical and laboratory side effects such as teratogenicity, hyperlipidemia and associated pancreatitis, leukopenia, thrombocytopenia, and transaminitis [2]. Although laboratory abnormalities are not observed very frequently, close laboratory monitoring is still a general practice for being on the safe side. In this study, we aimed to assess the side effects of isotretinoin on the laboratory parameters, and to detect the optimal frequency interval of laboratory monitoring for patients.

MATERIALS AND METHODS

One-hundred patients who started on oral isotretinoin therapy for acne vulgaris between January 1, 2018 and March 28, 2018 were retrospectively evaluated. Patients who have

received oral isotretinoin for at least 6 months were enrolled into the study. Liver transaminase levels (alanine aminotransferase [ALT], aspartate transaminase [AST]) and serum lipid profile (total cholesterol [T-CHOL], low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C] and triglyceride [TG]) were recorded for the baseline, 1-, 3- and 6- months of the therapy using electronic medical records. Patients who have higher levels than reference limits for liver transaminases and serum lipid profile at the baseline were excluded. Baseline laboratory levels of ALT, AST, T-CHOL, LDL-C, HDL-C and TG were compared with the 1st, 3rd and 6th month levels of same parameter. Comparisons for all parameters were also made between 1st, 3rd and 6th month levels. Reference limits were shown in Table 1. All patients took isotretinoin at a dose of 20, 30 or 40 mg daily at their first course or repeated courses. This study was approved by the Ethics Committee of the Hacettepe University and is registered under the following number 2020/18-19.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows v.23.0. (IBM Corp., Armonk, NY). Data were presented as means ± standard deviations (SDs) or medians (ranges) when appropriate. The demographic features of the study population were analysed using descriptive statistics. Categorical variables were expressed as frequencies and percentages. Generalised estimating equations (GEEs) were used to compare consecutive data of repeated measurements, including the baseline and 1-, 3- and 6-month ALT, AST, T-CHOL, LDL-C, HDL-C and TG levels. The level of statistical significance was set at $p<0.05$.

RESULTS

A total of 100 patients (25 male; 75 female) with a mean age of 22.39 ±4.70 years (range: 9 - 38 years) were enrolled into the study. The consecutive data of the mean ALT, AST, T-CHOL, LDL-C, HDL-C and TG levels are shown in Table 1. Baseline AST, T-CHOL, LDL-C and TG levels were found to be statistically lower than 1st, 3rd and 6th month levels ($p<0.001$, for all). There were no significant differences in AST, T-CHOL, LDL-C and TG between 1st, 3rd and 6th month levels ($p>0.05$, for all). The baseline HDL-C level was statistically higher than 1st month level ($p<0.001$) but there were no differences between baseline, 3rd and 6th month levels ($p>0.05$, for all). Figures 1 and 2 show the changes of laboratory parameters in relation to monthly intervals of isotretinoin treatment.

Table 1. Consecutive data on ALT, AST, T-CHOL, LDL-C, HDL-C and TG levels.

	Baseline	1th month	p_1	3th month	p_3	6th month	p_6	pα	pβ	p_γ
ALT (< 50 U/L)	15.30 ± 7.32 (6-42)	16.45 ± 11.63 (5-76)	0,939	15.77 ± 13.53 (7-132)	1.00	15,31 ± 9,41 (6-62)	1.00	1.00	1.00	1.00
AST (< 50 U/L)	18.99 ± 4.27 (10-33)	21.85 ± 6.38 (14-52)	**<0.001**	21.69 ± 8.55 (13-94)	**<0.001**	21,69 ± 6,57 (7-46)	**<0.001**	1.00	1.00	1.00
T-CHOL (< 200 mg/dL)	163.26 ± 30.45 (72-241)	173 ± 29.16 (88-227)	**0.001**	171.88 ± 30.80 (102-238)	**0.003**	179,4 ± 34,35 (75-241)	**<0.001**	1.00	0.121	0.062
LDL-C (< 130 mg/dL)	101.53 ± 21.74 (59-150)	110.98 ± 21.61 (56-167)	**<0.001**	111.65 ± 23.00 (65-181)	**<0.001**	114,76 ± 24,72 (53-178)	**<0.001**	1.00	0.088	0.417
HDL-C (>50 mg/dL)	51.80 ± 13.13 (28-119)	50.15 ± 12.42 (29-110)	**0.016**	49.37± 10.39 (33-84)	0.065	49,73 ± 12,22 (29-113)	0.069	1.00	1.00	1.00
TG (<150 mg/dL)	80.75 ± 37.75 (28-194)	93.14 ± 38.13 (21-211)	**<0.001**	98.08 ± 47.47 (23-260)	**<0.001**	97,76 ± 47,75 (26-268)	**0.001**	0.802	1.00	1.00

Data are presented as means ± standard deviations (minimum-maximum).

$p<0.05$, statistically significant and in bold

p_1 : p value between baseline and 1st month

p_3 : p value between baseline and 3rd month

p_6 : p value between baseline and 6th month

pα : p value between 1st month and 3rd month

pβ : p value between 1st month and 6th month

p_γ : p value between 3rd month and 6th month

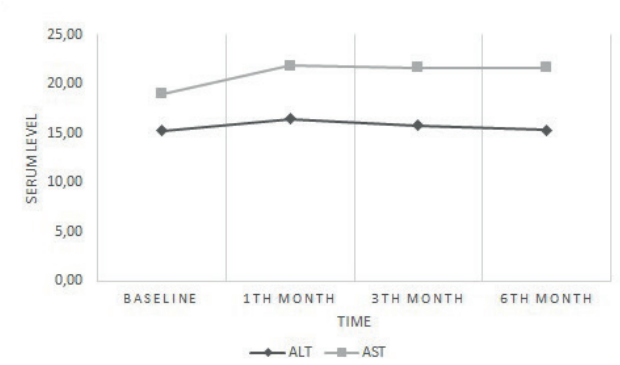

Figure 1. Changes in the mean ALT and AST levels during 6 months follow-up.

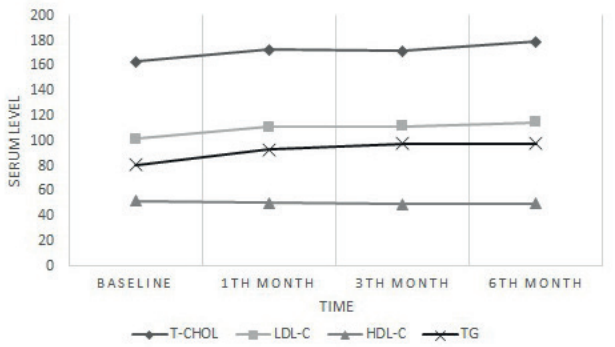

Figure 2. Changes in the mean T-CHOL, LDL-C, HDL-C and TG levels during 6 months follow-up.

DISCUSSION

Oral isotretinoin is considered as the most effective treatment available for severe acne. It is preferred by many dermatologists in severe cystic, nodular, or other actively scarring acne types [3]. Since the introduction of the drug for acne treatment, laboratory monitoring widely varied among prescribers without any standardized guidelines. The frequency of laboratory monitoring and the type of laboratory workup that should be performed during isotretinoin treatment have been recently changed as more data on the drug's side effect profile have been published.

Liver function tests, including ALT and AST, are routinely performed in acne patients receiving isotretinoin therapy. Many studies reported elevations in liver function tests, but these elevations were not associated with irreversible hepatic sequelae [2,4]. In the literature, mild to moderate ALT levels have been detected in up to 8.9% of asymptomatic patients. Fatty liver disease, as a result of obesity, and alcohol intake were identified as risk factors for elevated AST and ALT levels [4]. In our study, we found statistically significant elevation in AST levels, whereas there was no statistically significant elevation in ALT levels. The degree of elevation in AST levels was mild and no deviations from the reference range were observed. AST and ALT are enzymes that are not-specific for liver and they can be found in muscle and other tissues including red blood cells. Elevated muscle enzymes, including creatine kinase (CK), have been reported in many studies due to the intake of oral isotretinoin [5,6]. CK elevations greater than five times of normal can be a sign of rhabdomyolysis, which can lead to renal damage [7]. Webster et al.[8] claimed that AST elevation was usually accompanied by CK elevation, suggesting a muscle (rather than liver) source for the AST. They reported that ALT was less strongly paired with CK but there was still some overlap. Although we did not test CK to correlate it with AST and ALT in our study, we thought that the elevation of AST may be associated with muscle damage, not with liver damage. Therefore, Gamma glutamyltransferase (GGT), which is a liver specific enzyme, might be preferred for monitoring liver functions. Additionally, slight increase in AST level in the first month of treatment and its stability in the following months suggested that frequent follow-up was not required.

Serum lipid elevations are well known laboratory abnormalities seen in isotretinoin therapy [4]. Barbieri et al.[9] reported high TG levels (>500 mg/dL) in fewer than 1% of patients who were screened. High TG levels (>800 mg/dL) might be a risk factor for the development of acute pancreatitis but the few reported cases in the literature illustrate that pancreatitis is not likely caused by hypertriglyceridemia because elevations were mild to moderate in those patients, it occurs idiosyncratically more commonly than due to the hypertriglyceridemia, itself [10,11]. Therefore, screening the lipid profile might not be preventive for the development of acute pancreatitis. In our study, T-CHOL, LDL-C and TG were found to be statistically elevated on the 1st, 3rd and 6th month of therapy compared to the baseline but the following elevations were not statistically significant after the 1st month when compared with each other. This data might support that monitoring the serum

lipids is adequate just for the first month of therapy and there is no need for repeated tests. When examining the lipid and enzyme parameters, we did not take the variable doses of isotretinoin into account. This might be the limitation of the study.

Extending the monitoring intervals can reduce overcrowding in hospitals, especially under pandemic conditions. Thus, by not monitoring patients frequently, we can also treat patients who want to receive isotretinoin treatment during the pandemic period but are reluctant to come to the hospital very often.

In conclusion, mild elevations that are within the reference ranges might be expected in serum AST, T-CHOL, LDL-C, and TG levels during isotretinoin therapy, and the elevations are usually observed on the first month of therapy. There might be no need for close monitoring of AST, T-CHOL, LDL-C, and TG levels after the first month of therapy.

Author contribution

Study conception and design: DG, and EB; data collection: DG and EB; analysis and interpretation of results: DG, NA, BYA, SEE, SD, GEL, AK and EB; draft manuscript preparation: DG and EB. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Hacettepe University Ethics Committee (Protocol no. 2020/18-19/2020).

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Conflict of interest

The authors declare that there is no conflict of interest.

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ORIGINAL ARTICLE

Repetitive Transcranial Magnetic Stimulation in a Group of Treament-Resistant Obsessive-Compulsive Disorder Patients: A Descriptive Study

Müge Topcuoğlu[1]
ORCID: 0000-0002-7098-8806

Buket Cinemre[1]
ORCID: 0000-0001-6480-1454

Ali Erdoğan[1]
ORCID: 0000-0003-0329-6778

Naila Nabiyeva[1]
ORCID: 0000-0001-5698-4668

[1]Department of Psychiatry, Akdeniz University Medical Faculty, Antalya, Turkey.

Corresponding Author: Ali Erdoğan
Department of Psychiatry, Akdeniz University Medical Faculty, Antalya, Turkey.
E-mail: erdoganali006@hotmail.com

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ABSTRACT

Objective: This study aims to investigate the effects of repetitive transcranial magnetic stimulation (rTMS) treatment in a group of treatment-resistant obsessive-compulsive disorder (OCD) patients and to examine the relationship between various sociodemographic and clinical variables and treatment response.

Materials and Methods: Data including The Yale–Brown Obsessive Compulsive Scale (Y-BOCS) scores and various clinical and sociodemographic characteristics of 27 treatment-resistant OCD patients who received 30 sessions of low-frequency rTMS (LF-rTMS) treatment on the left dorsolateral prefrontal cortex (DLPFC) were analyzed.

Results: Mean Y-BOCS scores decreased significantly across week 0 and the 3rd week ($t(26)=10.59$, $p<.001$) and continued to decrease significantly across weeks 3 and 6 ($t(26)=11.47$, $p<.001$). 21(78%) patients were responders with at least a %25 decrease in the mean Y-BOCS scores, and 10(47.6%) of these 21 patients also met the complete response criteria with a 35% or more reduction in Y-BOCS. No significant difference was observed between responders and non-responders regarding various clinical and sociodemographic variables. The only reported side effects were headaches and local scalp tenderness, which improved in a short time.

Conclusion: This descriptive study has demonstrated the efficacy of a long-duration LF-rTMS application on the DLPFC in a group of drug-resistant OCD patients. This finding might contribute to the available literature, especially in drawing out a standardized treatment protocol in these cases.

Keywords: Neuromodulator, Obsessive-Compulsive Disorder, Transcranial Magnetic Stimulation, Treatment

INTRODUCTION

Obsessive-Compulsive Disorder (OCD) is a psychiatric disorder with a recurrent course in general, in which obsessions and/or compulsions are observed, leading to significant impairment in the functionality and quality of life of the individual [1]. The estimated lifetime prevalence of OCD in the general population is 2-3% [2]. The treatment guidelines published worldwide indicate the efficacy of both pharmacological and non-pharmacological treatments in OCD [3,4]. Medications such as selective serotonin reuptake inhibitors (SSRIs) or clomipramine and cognitive behavioral therapy (CBT) that include exposure and response prevention strategies are recommended as the first-line therapies in the treatment of OCD [5]. Treatment response in OCD is defined as a 35% or more decrease in the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) score compared to the baseline score [6, 7]. Criteria to assess treatment response in OCD include the use of at least 2 anti-obsessive drug treatments at the maximum recommended doses for at least 12 weeks and at least 20 hours of CBT [8, 9]. Partial response is defined as a reduction between 25% and 35% on the Y-BOCS; treatment resistance is defined as failure to respond to the above adequate treatment trial and less than 25% reduction on the Y-BOCS [6, 10, 11]. Literature shows that 20-30% of the patients do not respond clinically to the first-line treatments [10, 12]. In search of more treatment options, repetitive transcranial magnetic stimulation (rTMS) has been used in the treatment of OCD.

OCD has been associated with dysfunctions in the Cortico-Striato-Thalamo-Cortical (CSTC) circuits, including the Dorsolateral Prefrontal Cortex (DLPFC), Anterior Cingulate Gyrus, Supplementary Motor Area (SMA), Orbitofrontal Cortex (OFC), Medial Prefrontal Cortex (MPFC), and basal ganglia [13]. Neurophysiological studies have revealed that DLPFC, SMA, and OFC are hyperactive in patients with OCD. This hyperactivity is associated with motor planning and response control deficiencies and plays a role in the generation of ritualized behavioral responses and the regulation of negative emotional states such as fear and anxiety [14]. Hence, alternative treatment options are under investigation, especially those which directly affect OCD neurocircuits.

rTMS is a safe and non-invasive neuromodulatory method that uses repetitive magnetic waves to induce a depolarizing current in a localized region of the cerebral cortex. Magnetic pulses in rTMS can be delivered either at high (10-20 Hz) or low (<1 Hz) frequency. Low-frequency stimulation causes a decrease in neuronal activity, whereas high-frequency stimulation increases neuronal activity [15]. That is, TMS could be effective for OCD treatment by modulating cortical excitability and normalizing hyperactivity of the corticostriatal network. However, because TMS only temporarily alters cortical excitability, repetitive TMS is required when used for treating OCD. Indeed the evidence shows that rTMS is a safe and effective treatment strategy for drug-resistant OCD [16]. Successful treatment of OCD symptoms has been associated with a decrease in CSCT circuit hyperactivity produced by applying low-frequency rTMS (LF-rTMS) on the related cortical areas [17]. Despite all this evidence, the optimum TMS treatment protocol for OCD has not been established yet. Individual variations in response to TMS may perhaps influence this. There is also a lack of information about different demographic and clinical variables that may predict the response to rTMS in OCD patients [18]. Therefore, more research is needed to establish the optimal TMS treatment protocol (such as cortical target and stimulation frequency) for OCD [19].

The DLPFC, which is connected to the striatum, the anterior cingulate cortex, and the thalamus, is one of the most common targets for rTMS [20], and stimulation of this region can also affect connected areas, some of which are associated with OCD symptoms. While initial studies in the literature using rTMS on the DLPFC did not report superiority over placebo [6, 21], subsequent studies showed improvements in OCD symptoms between the active and sham groups [22, 23]. Due to conflicting results in the limited literature on the use of rTMS in the treatment of OCD and the heterogeneity in protocols, it is difficult to conclude whether rTMS is effective or not [16, 24]. Therefore, this study aims to expand the existing literature by evaluating the effects of left DLPFC targeted rTMS on various patients in the treatment of OCD. This study also retrospectively investigates the efficacy of rTMS in drug-resistant OCD patients treated with

rTMS and analyzes the relationship between the sociodemographic and clinical variables and the rTMS response.

MATERIALS AND METHODS

Study Design and Participants

Medical records of 36 patients treated with rTMS with a diagnosis of OCD at Akdeniz University Medical Faculty Department of Psychiatry between May 2019 and May 2020 were retrospectively analyzed. Four of the patients who underwent rTMS were excluded the study due to treatment discontinuation. In addition, 2 patients who previously received electroconvulsive therapy (ECT), 1 patient who was pregnant, and 2 patients with a history of neurological disorders were also excluded from the study. Except for the patients who were excluded from the study, 27 treatment-resistant OCD patients were found to be suitable for the study criteria and included in the study. Patients over the age of 18 with an OCD diagnosis according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) diagnostic criteria were included in the study. The patients also scored moderate to severe (scores of 16 and above) in Y-BOCS and did not respond to at least two anti-obsessive drug treatments of adequate dose and duration and thus were defined as treatment-resistant. The patients continued to take their medications during the four weeks before starting treatment and during the treatment. All patients were informed in detail about the treatment procedure and gave their written consents before rTMS. This study was conducted by the latest version of the Declaration of Helsinki and approved by the Akdeniz University Medical Faculty Clinical Research Ethics Committee (KAEK-865 / 11.11.2020).

Repetitive Transcranial Magnetic Stimulation (rTMS) Protocol

In the study, rTMS was applied using the Neuro-MS / D stimulator with eight shaped coils (Neurosoft, Ivanovo, Russia) by the updated safety guidelines [25]. The patients were comfortably seated on an adjustable chair in a semi-reclining position, with their heads placed on a head restraint and their arms on bilateral armrest. The patients' resting motor threshold was defined as the minimum stimulus intensity that produces a motor response during active contraction of the right abductor pollicis brevis muscle (APB) [26] and was determined using visual inspection of the relevant finger movement. After determining the resting motor threshold of the patients, the position of the coil was positioned on the anterior 5 cm along a parasagittal line from the optimum APB stimulation area to localize the DLPFC, which is the stimulation zone used for treatment [27]. After the coil is positioned and fixed on the stimulation area, the rTMS protocol was determined as 1200 pulses per session using a 1 Hz stimulation frequency, stimulation intensity at 100% of RMT. A total of 30 sessions of LF-rTMS was applied to each patient over the left DLPFC for six weeks, five days a week, excluding weekends.

Measurement and Assessment Tools

Patients included in the study were followed by clinical rating scales at regular time points, every three weeks from the first session to the end of the treatment. The Y-BOCS scale was applied to all patients on the day before the first rTMS session (0th week), on the 3rd week of rTMS treatment, and after 30 sessions of rTMS treatment (6th week) and clinical evaluation was performed. The Y-BOCS is the most widely used scale to assess the severity of OCD symptoms, equally weighing obsessions and compulsions, consisting of 10 items, each item graded between 0 and 4 points, and evaluated by the clinician [28]. The patients included in the study were evaluated according to the changes in Y-BOCS scores over 6 weeks. 35% or more reduction in Y-BOCS score from baseline was regarded as a complete response; A decrease of 25% or more in Y-BOCS score from baseline was classified as a partial response [10].

Statistical Analysis

Descriptive statistics were reported as percentage rates with frequency for categorical variables and mean (± standard deviation) or median for continuous variables. To compare independent groups, independent samples t-test was used in the case of normally distributed variables and the Mann-Whitney-U test for non-normally distributed variables. The Chi-Square test of independence was used to assess the relationships between categorical variables. Moreover, the repeated measures ANOVA test was used to evaluate the patients' mean YBOCS scores across three-time points (paired sample comparisons). Significance

was evaluated at $p \leq 0.05$ in the statistical analyses which were performed using the SPSS version 23.0.

RESULTS

Sociodemographic and Clinical Characteristics

Twenty-seven patients were included in this study, 59.26% (n=16) of whom were female. The mean age of the patients was 34.70 (SD=14.05, range= 19-66). The rate of the high school graduates (51.85%, n=14) was the highest in the study sample. Six patients (22.22%) were employed at the time of the study, and 16 (59.26%) were single. Twelve (44%) patients had a comorbid psychiatric illness, which was depression (30%, n=8), bipolar disorder (7.40%, n=2), and psychotic disorder (7.40%, n=2), respectively according to their frequency. The mean duration of illness in OCD patients 12.81 years (SD=10.41, range=2-38 years). Eighteen patients (66.67%) had comorbid nicotine addiction, whereas two patients (7.40%) met the alcohol use disorder criteria. The sociodemographic and clinical characteristics of the study group are summarized in Table 1.

Clinical Follow-up with the Yale-Brown Obsessive Compulsive Scale (Y-BOCS)

A repeated-measures ANOVA determined that mean YBOCS scores differed significantly between the three time points of assessment ($F(2, 52) = 206.82$, $p < .001$). Post hoc tests with the Bonferroni correction showed that the mean Y-BOCS scores decreased significantly across week 0 and the 3rd week ($t(26) = 10.59$, $p < .001$) and continued to decrease significantly across weeks 3 and 6 ($t(26) = 11.47$, $p < .001$). (Table 2 and Figure 1).

To figure out response rates, improvement in YBOCS scores were calculated as a percentage for every patient. Accordingly, 21 (78%) patients out of the 27 patients who showed at least a %25 decrease in the mean Y-BOCS scores compared to the baseline score (thus who also met the partial response criteria) were regarded as responders to rTMS treatment, whereas 10 (47.6%) patients in among these responders (N=21) also met the complete response criteria with at least %35 decrease in the mean YBOCS scores. The remaining 6 (N=27, % 22) patients in the study group were defined as non-responders.

No significant difference was found between the responders and non-responders compared to each other regarding various sociodemographics (gender, age) and clinical variables (illness duration, psychiatric comorbidity, suicide history) to evaluate the relationship between clinical response and these variables. (Table 3).

Side-effects of the rTMS Treatment

Five patients (18.52%) reported side effects after the rTMS treatment. Thus, three patients complained of headache and the other two patients complained of localized scalp tenderness. No significant difference was found between responders and non-responders in terms of the incidence of side effects (headache: $\chi2\ (1)=0.428$, $p=0.51$; localized scalp tenderness: $\chi2\ (1)=0.206$, $p=0.76$).

Table 1. Demographic and clinical characteristics of the patients.

Demographic and clinical characteristics	n	%
Gender		
Female	16	59.26
Male	11	40.74
Marital Status		
Single	16	59.26
Married	9	33.33
Divorced	2	7.41
Educational Status		
Primary education	6	22.22
Secondary education	14	51.85
Undergraduate education	7	25.93
Employment Status		
Employed	6	22.22
Unemployed	14	51.85
Student	4	14.81
Retired	3	11.11
Comorbidity/Comorbidities		
Yes	12	44.44
Depressive disorder	8	22
Bipolar disorder	2	7
Psychotic disorders	2	7
No	15	55.56
Smoking		
Yes	18	66.67
No	9	33.33
Alcohol Use Disorder		
Yes	2	7.41
No	25	92.59

*Descriptive statistics were reported as percentage rates with frequency for categorical variables. n: sample size.

Table 2. Comparison of mean Y-BOCS scores.

	Difference	*SE*	*df*	*t*	*p*
0th week Y-BOCS score - 3rd week Y-BOCS score	3.22	0.40	26	7.97	**< .001**
0th week Y-BOCS score - 6th week Y-BOCS score	6.52	0.62	26	10.46	**< .001**
3rd week Y-BOCS score - 6th week Y-BOCS score	3.30	0.44	26	7.44	**< .001**

*The repeated measures ANOVA test was used to evaluate the patients' mean YBOCS scores across three-time points (paired sample comparisons). To compare independent groups, independent samples t-test was used in the case of normally distributed variables. Significance was evaluated at p ≤ 0.05 in the statistical analyses. Y-BOCS: Yale-Brown Obsessive-Compulsive Scale, SE: Standard Error, df: Degrees of Freedom.

Table 3. Comparison of responders and non-responders.

	Response			*U*	*z*	*p*
	responders	non-responders	OR			
Female	11[12.44]	5[3.56]	0.23			.350
Male	10[8.56]	1[2.44]				
Smoking (+)	12[14.00]	6[4.00]	0.00			.071
Smoking (-)	9[7.00]	0[2.00]				
	Mean Rank					
Age	13.71	15.00		57.00	-0,35	.726
rTMS - power	13.57	15.50		54.00	-0.53	.598
rTMS – motor Treshold	13.55	15.58		53.50	-0.56	.578

Values are presented as numbers of patients (percentages of the sample).
*The Chi-Square test of independence was used to assess the relationships between categorical variables. Significance was evaluated at p ≤ 0.05 in the statistical analyses. rTMS: repetetive Transkranial Magnetic Stimulation, OR: Odds Ratio.

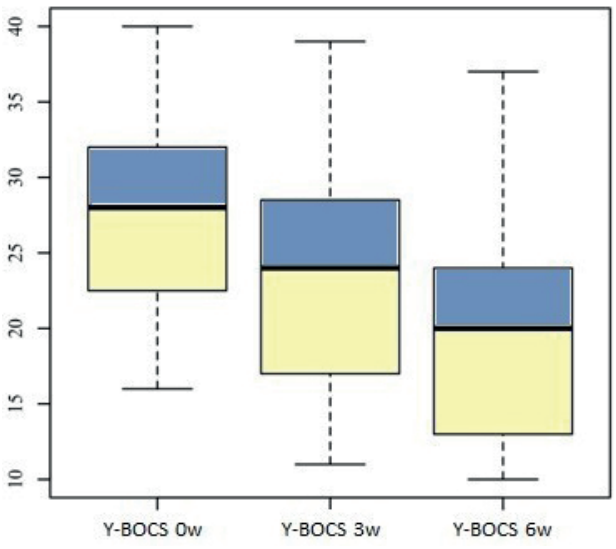

Figure 1. The changes of mean scores on Y-BOCS from the baseline to the third and sixth week of the rTMS treatment. Y-BOCS: Yale-Brown Obsessive Compulsive Scale; rTMS: repetitive transcranial magnetic stimulation. *p < 0.05 (Significance was evaluated at p ≤ 0.05 in the statistical analyses).

DISCUSSION

Data from studies on the role of rTMS in the treatment of OCD symptoms so far are limited. Moreover, most of the existing studies include small sample groups and target different cortical regions using different stimulation parameters [29, 30]. Therefore, the application of rTMS cannot be standardized and there is an increasing need for new studies addressing the possible use of rTMS as an efficacious therapeutic intervention for OCD.

To the best of our knowledge, this study is the first study conducted in Turkey on treatment-resistant OCD patients who underwent rTMS treatment. Our study showed the efficacy and safety of rTMS treatment in a group of treatment-resistant OCD patients. Treatment efficacy in this study was observed as a gradual decrease in YBOCS scores over time from the start to the completion of 30 sessions of rTMS treatment. 78% of the patients who received rTMS treatment responded to the rTMS treatment in general, whereas 37.03% met the "complete response" criteria. This finding is comparable to the findings reported in most previous studies with OCD patients in which the target application site was the DLPFC [31, 23]. For example, in a study by Sachdev et al., rTMS targeting the DLPFC was found to be effective in treatment-resistant OCD patients [23]. In addition, the recent evidence-based guidelines for the therapeutic use of rTMS in OCD patients indicate that LF-rTMS administered over the DLPFC may be efficacious [31]. And this supports our findings that LF-rTMS applied on DLPFC may be effective. Nevertheless, some studies report lower response rates to rTMS

treatment in OCD patients [32-34]. Different stimulation protocols or variations in the clinical characteristics of the patients might account for these inconsistent findings. For example, Prasko et al. [35] applied low frequency rTMS to the left DLPFC in their study, and no statistically significant difference was found between active and sham treatments. However, this may be attributed to the short-term (2 weeks) application of rTMS and the significant difference between the active and sham groups (the active group had higher initial YBOCS scores) in the initial YBOCS scores of the OCD patients included in their study. On the other hand, a recent network meta-analysis by Liang et al. showed that LF-rTMS applied to the DLPFC is more effective than sham rTMS. Moreover, in the same study, all rTMS treatment strategies were found to be similar to sham rTMS regarding tolerability [36].

The rTMS protocol used in a study might also be an important factor in treatment response to rTMS, and thus should be taken into consideration as well. Indeed, studies show that a short-duration protocol (1-2 weeks / 5-10 sessions) and a low stimulation intensity (80%) targeting DLPFC [32] are associated with poor response to rTMS. Conversely, rTMS was found to be effective when applied for longer durations (4-6 weeks / 20-30 rTMS sessions) and with a high stimulation intensity (100-120%) [1, 37]. The clinical heterogeneity and the variations between rTMS protocols make it difficult to draw a conclusion acceptable to everyone [24]. Therefore, it might help develop a standardized stimulation protocol to reduce the heterogeneity between studies that investigate rTMS effects in OCD. The high response rates observed in this study suggest that the stimulation protocol implemented here (LF-rTMS) is effective in treatment-resistant OCD patients. Although it might be unnecessarily long for research purposes, this protocol seems feasible for treatment success.

Comorbidity is common in patients with OCD [38]. Similar to the literature, a significant portion of the OCD patients included in this study had comorbidities (most commonly major depression). In some studies, it has been suggested that rTMS applied to the DLPFC causes improvements in comorbid anxiety and depression rather than specific OCD symptoms [6, 32, 39]. Therefore, rTMS applied to the DLPFC in our study may have produced improvements in OCD symptoms that were secondary to improvements in depression and anxiety. In addition, since the pharmacological treatments of the patients were continued during rTMS in this study, there may be a synergistic effect between rTMS and these drugs, which may affect recovery.

In the study group, no significant difference was found between responders and non-responders to rTMS treatment in terms of sociodemographic and clinical characteristics, similar to the previous findings reported in the literature [40, 41, 42]. In the study, headaches and localized scalp tenderness were the only complaints reported by three and two patients, respectively. Nevertheless, both of these complaints disappeared spontaneously within 3-4 days. No serious side effects such as seizures, acute psychiatric symptoms, or changes in cognitive functions were observed in patients. These results are consistent with most of the previous findings reported in rTMS studies [22, 43] and support the application of rTMS targeting the DLPFC as a safe and well-tolerated treatment modality in OCD patients.

However, this study has some limitations. First, the study is a retrospective study with a relatively small sample size and no control group. Hence, it is difficult to exclude the placebo effect and generalize the results of the study. Although no change was made in medication type or dosing, we should bear in mind that patients continued their medications which might also have an effect on the treatment response. Thus, further studies which keep out medication effects are needed to clarify this problem.

CONCLUSION

In conclusion, this study provides evidence that rTMS targeting DLPFC is an effective method in the treatment of drug-resistant OCD patients. Besides, it has been demonstrated that rTMS has a low side-effect profile. Large-scale RCTs will provide a better understanding of this method with regard to establishing a standardized rTMS protocol for OCD treatment in clinical practice.

Author contribution

Study conception and design: AE, BC, MT, NN; data collection: MT, NN; analysis and interpretation of results: BC, AE; draft manuscript preparation: AE, BC, MT, NN. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Akdeniz University Medical Faculty Clinical Research Ethics Committee (Protocol no. KAEK-865 / 11.11.2020).

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Conflict of interest

The authors declare that there is no conflict of interest.

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ORIGINAL ARTICLE

Non-Wilms' Renal Tumors In Childhood

Burak Ardıçlı[1,2]
ORCID: 0000-0002-1630-7029

Saniye Ekinci[1,2]
ORCID: 0000-0002-2961-7069

Arbay Özden Çiftçi[2]
ORCID: 0000-0001-9226-764X

Feridun Cahit Tanyel[2]
ORCID: 0000-0002-8301-3012

Diclehan Orhan[3]
ORCID: 0000-0003-3637-5392

Canan Akyüz[4]
ORCID: 0000-0002-7105-4191

İbrahim Karnak[2]
ORCID: 0000-0003-2185-3902

[1]Hacettepe University, Institute of Health Sciences, Department of Medical and Surgical Research, Ankara, Turkey.

[2]Hacettepe University, Faculty of Medicine, Department of Pediatric Surgery, Ankara, Turkey.

[3]Hacettepe University, Faculty of Medicine, Department of Pediatrics, Pediatric Pathology Unit, Ankara, Turkey.

[4]Hacettepe University, Faculty of Medicine, Department of Pediatrics, Pediatric Oncology Unit, Ankara, Turkey.

Corresponding Author: Burak Ardıçlı
Hacettepe University, Faculty of Medicine, Department of Pediatric Surgery, Ankara, Turkey.
E-mail: burakardicli@gmail.com

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ABSTRACT

Objective: To determine the outcomes, demographics, clinical and surgical characteristics of NWRT in children.

Methods: Forty-three children who underwent surgery for NWRT between 1970 and 2016 were included. The charts and surgery notes were evaluated retrospectively and age, sex, complaints and physical findings at presentation, results of biochemical tests, details of surgery, pathologic findings, and the postoperative course were noted.

Results: The female to male ratio was 15:28. Distribution according to histological groups were CCSK (n=14), CMN (n=10), RCC (n=7), CN (n=4), angiomyolipoma (n=2), MRT (n=2), sclerosing nephrogenic rest (n=1), renal tubular adenoma (n=1), metanephric stromal tumor (n=1), and renal adenocarcinoma (n=1). Nephroureterectomy was the most frequently used surgical treatment (n=38) (88%). The mortality rate was 21% in malignant NWRT (3 with CCSK, 2 with MRT) and 10% in benign NWRT (2 CMN) during the five-year follow up period.

Conclusion: Non-Wilms renal tumors are rare in childhood. The presumptive diagnosis is usually a Wilms tumor. Hypercalcemia may be encountered. A nephroureterectomy is necessary in most cases. NSS can be performed in selected cases and under suitable conditions.

Keywords: Renal tumor, congenital mesoblastic nephroma, clear cell sarcoma, malignant rhabdoid tumor, cystic nephroma, multilocular cyst of the kidney

INTRODUCTION

The Wilms tumor is the most common malignant tumor of the kidney, whereas non-Wilms' renal tumors (NWRT) are quite rare in childhood. There are several histopathological diagnoses in this group such as clear cell sarcoma of the kidney (CCSK), congenital mesoblastic nephroma (CMN), renal cell carcinoma (RCC), cystic nephroma (CN), angiomyolipoma, and other less common tumors [1,2]. These tumors are poorly understood due to their heterogeneity and rarity. Therefore, a retrospective clinical study was conducted to present a clear picture of the entire spectrum of pediatric NWRT based on our experience and a brief literature review. The current study consists of one of the largest non-collected pediatric NWRT series treated in a single medical center.

MATERIALS AND METHODS

The records of children treated for NWRT at our department between 1970 and 2016, inclusive, were reviewed retrospectively. Information recorded for each patient included age at admission, gender, past medical and family history, presenting signs and symptoms, clinical characteristics, results of laboratory tests (complete blood counts, serum electrolytes levels, creatinine levels, and urine tests), radiologic examination methods (US, IVP, CT, MRI), stage of the disease, preoperative chemotherapy and radiotherapy applications, preoperative biopsy results, details of surgical intervention (nephroureterectomy, partial nephrectomy, enucleation of tumor, and excision of palpable lymph nodes), early and late complications of surgery, pathologic findings, postoperative chemotherapy and radiotherapy applications, and outcomes.

Data were analyzed with SPSS 23.0 software (Macintosh, IBM Corp., Armonk, NY). Descriptive data are expressed as frequency and percentage and continuous variables as medians, interquartile ranges (IQR) and minimum and maximum values. For the survival outcomes, Kaplan–Meier method was used. This study was approved by the Institutional Ethical Committee (GO 16/745).

Surgery details

If the excision of the tumor could be completed without a high level of risk, surgery was recommended for all patients as the initial mode of treatment. Otherwise, preoperative chemotherapy was recommended in patients whose initial imaging findings consisted of a primary malignant renal tumor. The term nephron sparing surgery (NSS) refers to a partial nephrectomy, wedge resection, or enucleation. A partial nephrectomy is performed by excision of the upper or lower pole that contains the tumor. Enucleation is performed by excision of the small lesion with a thin rim of surrounding renal parenchyma, if possible.

RESULTS

There were 43 children who underwent surgery for NWRT during the study period. The male to female ratio was 1.8 (28 boys and 15 girls). The median age at diagnosis was 30 months (15 days–16 years). The distribution according to histological diagnosis was CCSK (n=14, 33%), CMN (n=10, 23%), RCC (n=7, 17%), CN (n=4, 9%), angiomyolipoma (n=3, 5%), malign rhabdoid tumor (n=2, 5%), sclerosing nephrogenic rest (n=1, 2%), renal tubular adenoma (n=1, 2%), metanephric stromal tumor (n=1, 2%), and renal adenocarcinoma (n=1, 2%) (Table 1). The presenting symptoms were abdominal swelling (n=16, 37%) and abdominal pain (n=10, 23%). Other presenting symptoms included hematuria (n=7, 16%), weakness (n=2, 4%), respiratory distress (n=1, 2%), and vomiting (n=1, 2%). A tumor was detected incidentally in four (9%) and prenatally in one (2%). One patient with RCC also had tuberous sclerosis with no family history. The most common

Table 1. Demographics and Clinical Characteristics of Children With Non-Wilms' Renal Tumors

Sex	**n**	**%**
Male	28	66
Female	15	34
Age (year)		
0	8	18
1–4	20	46
5–9	9	20
10–14	7	16
15–19	-	-
Tumor histology		
Clear cell sarcoma of the kidney	14	33
Congenital mesoblastic nephroma	10	23
Renal cell carcinoma	7	17
Cystic nephroma	4	9
Angiomyolipoma	2	5
Malignant rhabdoid tumor	2	5
Sclerosing nephrogenic rest	1	2
Renal tubular adenoma	1	2
Metanephric stromal tumor	1	2
Renal adenocarcinoma	1	2
Tumor stage		
Local (stage1–2)	14	32
Regional (stage 3)	18	42
Distant (stage 4)	11	26
Surgery approach		
Nephroureterectomy	38	88
Lymph node sampling	10	23
Nephron sparing surgery	4	9
Subtotal excision	1	2
Recurrence	6	14
Distant metastasis	11	25
Mortality	7	16

finding upon physical examination was a palpable abdominal mass (n=37, 86%). Other findings were hypertension (n=3, 7%), paleness (n=2, 4%), tachycardia (n=2, 4%), growth retardation (n=1, 2%), and tachypnea (n=1, 2%). Preoperative laboratory investigations revealed that seven patients (16%) (4 with CCSK, 1 with CMN, and 2 with RCC) had hematuria, two patients (4%) had anemia (CMN, MRT), and three patients (7%) had hypercalcemia (2 with RCC, 1 with CMN). The tumor location on either the right or left side was 22 and 21, respectively. No bilateral tumor was identified.

A Tru-Cut needle biopsy was used in seven patients because either the radiological findings were not suggestive of a Wilms tumor, or there was no response to neoadjuvant chemotherapy for a Wilms tumor. Histopathological findings were suggestive for RCC (n=3), CCSK (n=2), neuroblastoma (n=1), MRT (n=1), and mesenchymal tumor (n=1). The final diagnose changed to CCSK in two patients (one with neuroblastoma and the other with a mesenchymal tumor diagnosis) after nephroureterectomy. Frozen biopsies were performed in 7 (16%) to determine if NSS could be performed. These biopsies were reported as malignant renal tumors in five children (final diagnoses were RCC in 2, CMN in 1, CCSK in 1, and angiomyolipoma in 1). The remaining two biopsies revealed benign or equivocal findings, which were subsequently reported as RCC and sclerosing nephrogenic rest, respectively. Therefore, the patient with RCC experienced reoperation for complementary nephroureterectomy.

Distant metastases were detected in 10 patients (23%) having CMN (n=4), CCSK (n=4), RCC (n=1), and renal adenocarcinoma (n=1). Bone metastases were detected in 7 (4 with CCSK, 2 with CMN, and 1 with renal adenocarcinoma), lung metastases in two children (RCC and CMN) who also underwent metastasectomy, and liver metastases in 2 (renal adenocarcinoma and CMN).

The postoperative period was uneventful in all. Seven patients (16%) died and five patients (11%) were lost to follow up. One of the patients with CCSK was operated on for an adhesive intestinal obstruction. Two patients with CCSK (n=2) were reoperated on due to local recurrence. Patients with MRT had the highest mortality rate. Multiple metastases were the cause of death in four children. The five-year OS were 76% in malignant NWRT (Fig. 1).

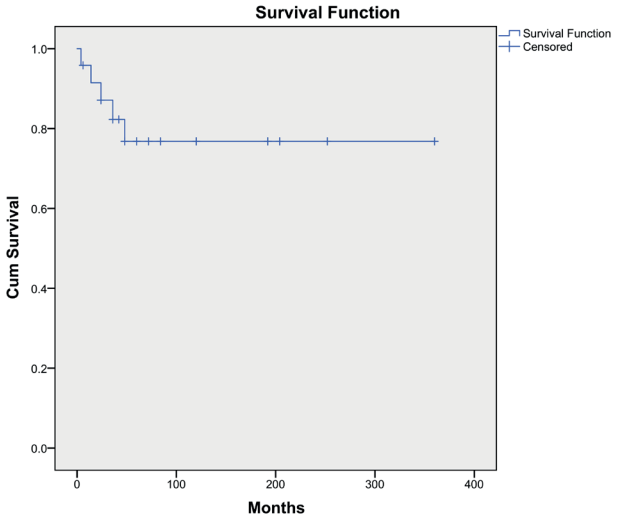

Figure 1. Overall survival for patients operated with malignant NWRT

Clear cell sarcoma

Fourteen patients were diagnosed with CCSK. The male to female ratio was 3.6. The median age at diagnosis was 30months (18-72). The presenting symptoms were abdominal pain (n=5, 35%), abdominal swelling (n=4, 28%), hematuria (n=4, 28%), and malnutrition (n=1, 7%). A palpable abdominal mass was encountered in all. The median size of the mass was 128mm (IQR, 105–150). The tumor was located in the right kidney in most (n=8, 57%). No familial or syndrome-associated CCSK was identified. The tumor stages were as follows: stage 1 (n=1, 7%), stage 2 (n=8, 58%), stage 3 (n=3, 21%), and stage 4 (n=2, 14%). Preoperative Tru-Cut biopsies were performed in three and the presumptive diagnoses were CCSK, neuroblastoma, and malignant round cell tumor. All patients underwent a nephroureterectomy (Table 2). Excisions of lymph nodes were performed in five and the lymph nodes were positive for metastasis in one. This patient had local recurrence one year later. A frozen biopsy was performed in one. Distant metastases were detected in four (28%) and bone was the target tissue in all. Local recurrence was seen in two having bone metastases at the time of diagnosis and these patients underwent reoperation 6 months and 1 year after the first operation. Neoadjuvant chemotherapy was given to five patients (Adriamycin was included in three cases) and adjuvant chemotherapy was used in eight. Eight patients were treated with adjuvant radiotherapy. The median follow up duration was 48 months (IQR, 36-84). Three patients (2 with stage 4, 1 with stage 2) died within two years (Table 3).

Table 2. Summary of The Management of Patients

	CCSK (n)	CMN (n)	RCC (n)	CN (n)	Angiomyolipoma (n)	MRT (n)	Others (n)
	14	10	7	4	2	2	4
Neoadjuvant chemotherapy	5	3	1	1	-	1	-
Surgery							
Nephroureterectomy + Lymph node sampling	5	2	1	-	1	1	-
Nephroureterectomy	9	8	5*	4	-	-	1
Subtotal nephrectomy	-	-	-	-	-	1	-
Partial nephrectomy	-	-	1†	-	-	-	1
Enucleation	-	-	-	-	1	-	1
Complementary nephroureterectomy	-	-	1†	-	-	-	-
Adjuvant chemotherapy	14	4	4	-	-	2	1
Adjuvant radiotherapy	8	1	1	-	-	1	-
Palliative chemotherapy	3	2	-	-	-	2	-

Note: CCSK, clear cell sarcoma of the kidney; CMN, congenital mesoblastic nephroma; RCC, renal cell carcinoma; CN, cystic nephroma; MRT, malignant rhabdoid tumor; *Frozen biopsy in 2 cases, †Complementary nephroureterectomy after partial nephrectomy.

Table 3. Characteristics of Patients Who Died of Disease

Case no	Sex	Age (years)	Metastasis and recurrence	Surgical intervention	Pathology	Follow up (year)
1	M	2.5	Bone, local recurrence	Nephroureterectomy (complementary*)	CCSK	4
2	M	1	Bone	Nephroureterectomy + LN sampling	CCSK, renal sinus invasion (+), LN (-)	5
3	M	3	Local recurrence	Nephroureterectomy	CCSK, capsule invasion (+)	2
4	F	15 days	Liver, lung, recurrence in the liver	Nephroureterectomy + adrenalectomy + tumor enucleation from liver + LN sampling	Cellular CMN, adrenal (+), liver (+), LN (-)	3
5	M	4	Bone, local recurrence	Nephroureterectomy	Cellular and spindle cell CMN, capsule invasion (-)	2
6	M	5.5	Local recurrence	Nephroureterectomy	MRT, capsule invasion (+), LN (+)	1
7	M	1.2	-	Subtotal nephrectomy	MRT, capsule invasion(+)	1 month

Note: CCSK, clear cell sarcoma of the kidney; LN, lymph node; CMN, congenital mesoblastic nephroma; MRT, malignant rhabdoid tumor; *Underwent incisional biopsy previously at another center.

Congenital mesoblastic nephroma

Ten patients were diagnosed with CMN. The male to female ratio was 2.3 with the median age of 6 months (IQR, 1-36) at diagnosis. The presenting symptoms were abdominal swelling (n=4, 40%), vomiting (n=1, 10%), anemia (n=1, 10%), prenatally detected abdominal mass (n=1, 10%), respiratory distress (n=1, 10%), and hematuria (n=1, 10%). The diagnosis was made incidentally in two (20%). The findings on physical examination were abdominal mass (n=10) and hypertension (n=3). One patient had hypercalcemia. The median size of the mass was 92 mm (IQR, 62-135). The tumor was predominantly located in the right kidney (n=7, 70%). No familial or syndrome-associated CMNs were identified. Distant metastases were detected in five at the time of diagnosis, bone (n=2), lung (n=2), and liver (n=1). The tumor stages were as follows: stage 1 (n=4, 40%), stage 2 (n=1, 10%), stage 3 (n=1, 10%), and stage 4 (n=3, 30%). An incisional biopsy had been performed before admission on one patient at another center. No Tru-Cut biopsy was performed in the CMN. All patients underwent nephroureterectomy and excision of palpable lymph nodes was performed in two (Table 2). A perioperative frozen biopsy was used in one. An adrenalectomy and enucleation of hepatic metastasis were performed in one. Histopathological evaluation revealed cellular type in 8 and classic in two. Local recurrence occurred in two. One of these patients had undergone hepatic enucleation and also had a relapse in the liver. The other patient had bone metastasis at the time of diagnosis. The patient was reoperated

on for local recurrence and given chemotherapy and radiotherapy. However, the patient died after two years. Neoadjuvant chemotherapy was used in three and adjuvant chemotherapy was given to four. Adjuvant radiotherapy was given in two having tumor invasion of the renal capsule and lymph node. The other had a local recurrence during follow up without treatment in the postoperative fifth month. The histopathological features of the recurrent lesion were cellular CMN. Both patients died within two years (Table 3). The mean follow-up duration was 48 months (IQR, 24-156) in all CMN patients.

Renal cell carcinoma

RCC was diagnosed in seven. The male to female ratio was 0.75 and median age at diagnosis was 108 months (60-156). The presenting symptoms were abdominal pain (n=3, 42%), hematuria (n=2, 28%), and abdominal swelling (n=1, 14), and the diagnosis was incidental in one (14%). The patient with the incidental diagnosis also had tuberous sclerosis complex. The abdominal mass was detected during physical examination in four (57%). Two patients had hypercalcemia. The median size of the tumor was 59 mm (IQR, 57-60) and the tumor was located in the left kidney in five. The distribution of patients according to stages were stage 1 (n=3), stage 3 (n=3), and stage 4 (n=1, lung). A preoperative Tru-Cut biopsy was performed in two. A frozen biopsy was performed in three who had a mass on only one pole of the kidney. Frozen biopsies revealed RCC (n=2) and benign pathology (n=1). After evaluation of the permanent sections, the patient with benign pathology was found to be RCC. A complementary nephrectomy was performed in the patient. With the exception of this patient, all others underwent a nephroureterectomy at initial surgery and a lymph node excision was performed in only one (Table 2). The histopathological diagnoses were papillary RCC (n=5), clear cell tumor (n=1), and chromophobe RCC (30%)+oncocytoma (70%) (n=1). Distant metastasis to the lung was detected in one and treated with a metastasectomy. Chemotherapy was given in five and radiotherapy was given in one. No recurrence was seen. Three patients were lost on follow up. The median follow up duration was 60 months (IQR, 60-120) for four patients. They are still under follow up and disease free.

Cystic nephroma

Four patients were diagnosed with CN. The male to female ratio was 1. The median age at diagnosis was 15 months (IQR, 12-19). The presenting symptom was abdominal swelling in three (75%) and one was diagnosed incidentally (25%). A palpable abdominal mass was encountered in all patients as the sole finding. The median size of the lesion was 100mm (IQR, 30–120). The right kidney was predominantly affected (75%). Nephroureterectomy was performed in all patients (Table 2). The median duration of follow up was 96 months (IQR, 60-132). All are still disease free and being followed up.

Angiomyolipoma

There were two patients with angiomyolipoma, one male and one female aged 6.5 and 10 years, respectively. The presenting symptoms were abdominal pain (n=1) and enuresis (n=1). Physical examination revealed an abdominal mass in both cases. The sizes of the lesions were 200mm and 130mm in the right and left kidney, respectively. A partial nephrectomy with a frozen biopsy was performed in one (Table 2). A Tru-Cut biopsy was performed in the other patient, who had a 130 mm mass found to be a mesenchymal tumor. Therefore, a nephroureterectomy and excision of the palpable lymph nodes were performed in this patient (Table 2). The final diagnosis was angiomyolipoma. The patients are still disease free after 7 years and 11 years of follow up.

Malignant rhabdoid tumor

MRT was diagnosed in two male patients 1.2 and 5.6 years of age. The presenting symptoms were abdominal pain and swelling. The physical examination revealed abdominal distension and abdominal mass. The size of both left-side lesions was 120 mm. No distant metastasis was detected. A Tru-Cut biopsy had been performed on one (5.6 years old) at another center and was reported as MRT. Neoadjuvant chemotherapy was given to the patient. A nephroureterectomy was performed on this patient. In the other patient, a subtotal nephrectomy could be performed due to dense adhesions (or local invasion) (Table 3). Only the patient who underwent the nephroureterectomy received adjuvant chemotherapy. However, a

recurrent lesion of 7 cm in size was detected in the left paravertebral region during chemotherapy in the postoperative 7th month. A Tru-Cut biopsy of the recurrent lesion was reported as MRT. The patient died in the postoperative 12th month. The other patient developed sepsis and died in the pediatric intensive care unit on the postoperative 21st day (Table 3).

Renal tubular adenoma

A 14-year-old boy presented with abdominal pain. The physical examination was normal. A mass of 27 mm in diameter in the left kidney was detected by US. A partial nephrectomy was performed and the histopathological examination revealed renal tubular adenoma. The patient was lost to follow up.

Sclerosing nephrogenic rest

A 6-year-old girl presented with enuresis. A mass originating in the left kidney was detected by US. The size of the mass was 25mm and contained cysts and calcification. Enucleation with a frozen biopsy was performed. The histopathological examination revealed a sclerosing nephrogenic rest. The follow up duration has been 10 years and the patient is still being followed without disease.

Metanephric stromal tumor

A 3.5-month-old boy had been admitted to another center with abdominal pain and swelling. The physical examination showed an abdominal mass and CT revealed a lesion (120 mm) located in the left kidney. He was operated on and an incisional biopsy previously revealed a spindle cell tumor. A nephroureterectomy was performed after neoadjuvant chemotherapy at our center. A histopathological examination revealed a metanephric stromal tumor. The follow up duration was 120 months and he is still being followed without disease.

Renal adenocarcinoma

A 3-month-old boy presented with abdominal swelling due to an abdominal mass. Ultrasonography and IVP revealed a lesion in the right kidney (120 mm). A nephroureterectomy and liver wedge biopsy were performed. The histopathological examination revealed renal adenocarcinoma. Distant metastases were detected in the bones and liver, and the patient was evaluated as stage 4. The patient received adjuvant chemotherapy and was followed up for 180 months. He is still under follow up and disease free.

DISCUSSION

Although non-Wilms' renal tumors are rare in childhood, they present a large spectrum of pathologic diagnoses and have various histological subtypes which closely relate to associated morbidities and mortality [3,4].

These patients presented with abdominal swelling, abdominal pain, and hematuria. None of these signs and symptoms is specific or helpful in the differential diagnosis of a renal mass. Therefore, the surgeon must take into consideration the findings on physical examination and results of the radiological examination to evaluate the resectability of the tumor.

About two-thirds of infantile abdominal masses are of renal origin. CMN is the most common renal tumor in infants. In the present series, the most common diagnosis was CMN in patients both until 12 months of age and under 5 years of age, 87.5%-35%, respectively. On the other hand, almost half of the patients with NWRT are under 5 years of age (Table 1). With regard to the age distribution of diagnosis, our findings are in agreement with the figures reported in the literature [5-7]. There were no patients over 15 years of age in the present series.

Hypercalcemia is a rare metabolic disorder associated with childhood cancers. Hypercalcemia was detected in two patients with RCC and one with CMN. A study of 2400 solid tumors showed only 17 cases with hypercalcemia (0.7%). Of the 325 children with renal tumors in the same study, only four (1.2%) had hypercalcemia (MRT, CMN) [8]. Another study showed that parathormone levels are high in approximately half of the NWRT patients having hypercalcemia. However, CCSK with bone metastases shows no association with hypercalcemia [9]. Therefore, hypercalcemia associated with RCC and CMN might be a kind of paraneoplastic syndrome in which the mechanism is not clearly understood.

Distant metastasis was detected in 32% of malignant NWRT. The most common metastasis site was bone (63%), and half the patients with bone metastasis had CCSK. On the other hand, patients with CCSK (71%) or RCC (14%) in local stages, cellular CMN (50%), and MRT (50%) presented distant metastases. Except for CMN, our findings were in agreement with the figures reported in the literature [7,10]. CMN is usually a benign tumor, but distant metastases have been reported in cellular CMN where local recurrence occasionally occurs, and the main site of the metastasis is the lung. In addition, other studies suggested positive surgical margins as the only parameter that predicts recurrence [11,12]. Most of the CMN patients had a cellular subtype and two had a recurrence due to local spillage. A high rate of cellular subtype histology and incomplete eradication of the tumor may explain the high rate of metastasis and local recurrences, respectively, in the present series.

Clear cell sarcoma of the kidney had previously been considered an unfavorable histological variant of the Wilms tumor. However, it has been evaluated as a separate tumor other than Wimls tumor [6,13,14]. CCSK comprises 2%–5% of all primary renal malignancies in children [15]. Of the patients with localized disease, metastatic disease is encountered in 6%–7% of patients at diagnosis [15,16]. CCSK is the most common tumor in this series with a presenting mean age of 3.8 years and with a male gender predominance. All patients presented with a large abdominal mass, half also had hematuria, and one third had bone metastasis similar to previously reported rates [2,6,7,17]. Although hypertension had been reported in 40%–65% of patients with CCSK, none of the patients had hypertension in the present series [17,18]. Preoperative Tru-Cut biopsy results may not be confirmative and the initial diagnosis may be different than CCSK. Nephroureterectomy and lymph node sampling is the primary surgical treatment [15]. The Children's Oncology Group (COG) recommends immediate surgical excision if it can be performed safely. SIOP recommends preoperative chemotherapy with actinomycin and vincristine in local disease and three drugs (including doxorubicin) in metastatic disease for children between 6 months and 16 years [15,16,19]. However, its effect cannot be predicted and the size of lesion may not change after Adriamycin-containing chemotherapy.

Congenital mesoblastic nephroma is the most common renal neoplasm in the first year of life, particularly in neonates [2,20]. The age at the time of diagnosis was less than one year in 87.5% in NWRT in our series. CMN composed 70% of the NWRT diagnosed at less than 1 year of age and more than half the patients were diagnosed in the neonatal period. In spite of adverse reports, the review of several patients has suggested a male predominance in our series [21-24]. Presenting signs and symptoms and their proportional frequency, such as abdominal mass (100%), hypertension (30%), hypercalcemia (10%), and hematuria (10%) did not differ from previous reports [24-26]. Recurrence and/or metastatic disease can occur in cellular CMN and cause a high mortality rate. The patients require close follow up for 12 months after surgery [27]. Surgery is the mainstay treatment of recurrent and metastatic lesions and chemotherapy may not affect the outcome. The most frequent sites of metastasis are the lung and the liver. About half of patients with recurrence and/or metastasis die of the disease [27]. A higher rate of recurrence and mortality (20%) occurred in our two patients.

Renal cell carcinoma is an unusual tumor in childhood, presenting at 9–15 years of age [28]. RCC can be seen in childhood cancer survivors and genetic syndromes such as tuberous sclerosis, von Hippel-Lindau disease, familial clear cell renal cancer, hereditary papillary renal carcinoma, hereditary leiomyomatosis, and in patients with end-stage or cystic renal diseases, sickle cell hemoglobinopathies, and child kidney transplant recipients [29]. There were two patients, one with tuberous sclerosis complex (TSC), under the age of five in our series. The others were over the age of nine. Tuberous sclerosis complex is an autosomal dominant disorder with characteristic tumors involving multiple organ systems. Angiomyolipoma is common in TSC, however RCC can also be encountered in the kidney rarely. On the other hand, TSC associated RCC are diagnosed at a younger age, as seen in our series [30]. No gender dominance was observed in children, but male predominance was encountered in the present series, as in adults [10,31-33]. The usual triad of hematuria (28%), abdominal pain (42%), and palpable mass (57%)

were seen in two patients (28%) in the present series as in previous reports [15]. Paraneoplastic manifestations can be seen in RCC such as hypercalcemia, hyperglycemia, renin production, prolactin production, hepatic syndromes, hematologic syndromes, and neuromuscular syndromes, and the clinical implications of these RCC-related paraneoplastic syndromes are not well established [34]. Hypercalcemia is present in up to 20% of patients [35]. Hypercalcemia was encountered in two cases (28%) in the present series. The main treatment is radical nephrectomy with the excision of regional lymph nodes. The need for a complete retroperitoneal lymph node dissection is controversial [29]. The rate of metastatic disease at the time of RCC diagnosis is approximately 20%, and similarly one patient with pulmonary metastasis was encountered in the present series [15]. All but one of our patients with RCC underwent a nephroureterectomy. A complementary nephroureterectomy was performed following the histopathological diagnosis of RCC in one patient undergone a partial nephrectomy during surgery. Although a partial nephrectomy has been recommended for RCC lesions smaller than 4 cm, a nephroureterectomy seems much safer due to the possibility of a multifocal tumor [2]. Immunological therapy and radiotherapy were given in three patients (with stage 3 and 4). Although four patients are still under follow up and disease free, discussion on the survival rate in RCC could not be included in this series due to the loss of follow up of others.

The malignant rhabdoid tumor is a malignant childhood renal tumor and is associated with a poor prognosis [36]. Unfortunately, both patients with MRT died in the present series, one with late postoperative sepsis and the other with recurrent disease.

The term cystic renal tumor covers cystic nephroma (CN) or multilocular cysts of the kidney, localized renal cystic disease, cystic partially differentiated nephroblastoma (CPDN) or cystic WT in childhood. The distinguishing characteristics of CN are a fully cystic mass and numerous thin-walled septa covered by epithelium without blastemal and other embryonic elements [2,37]. A nephrectomy is the surgery of choice in large CNs. However, if the radiological data suggest a CN in a small-sized renal cystic mass, NSS may be an option after confirmation of the benign nature of the lesion with a frozen histopathological examination, if suitable. Otherwise, a nephrectomy would be curative.

Renal angiomyolipoma is a benign renal tumor. This tumor may be associated with TSC or can occur as a component of sporadic lymphangioleiomyomatosis [38]. Asymptomatic small angiomyolipomas (<4cm) can be followed up with US, CT, or MRI, while symptomatic or large lesions should be treated with NSS or embolization [29]. The rate of angiomyolipoma is 4% in our series without association with TSC.

A nephrogenic rest that is persistent metanephric blastemal tissue in the kidney after the 36th week of gestation associates with the Wilms tumor. The majority of nephrogenic rests disappear spontaneously as the incidence of nephrogenic rest is about 100 times greater than the Wilms tumor. Nephrogenic rests are histologically classified as incipient nephrogenic rests, sclerosing nephrogenic rests, and hyperplastic nephrogenic rests. In addition, when most nephrogenic rests are defined, sclerosis develops [2]. In the present series, one patient had a sclerosing nephrogenic rest.

Metanephric stromal tumor (MST) is a benign stromal tumor of the kidney, and the most common presentation was an abdominal mass. MST is thought to be a biphasic tumor that can be merged with Wilms tumor histology. A relationship with papillary renal cell carcinoma has been reported [39]. A nephrectomy is usually curative [29].

CONCLUSION

Non-Wilms' renal tumors are rare in childhood and half are malignant. Presumptive diagnosis is usually the Wilms tumor. Hypercalcemia may be encountered in patients with CMN and RCC. Nephroureterectomy is necessary in most cases. NSS can be performed in selected cases and under suitable conditions. The OS rate is 76% in malignant NWRT.

Author contribution

Study conception and design: BA, SE, and İK; data collection: BA and SE; analysis and interpretation of results: BA, SE, and DO; draft manuscript preparation: BA, SE, AOC, FCT, DO, and İK. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Institutional Ethical Committee (Protocol no. GO 745/2016).

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Conflict of interest

The authors declare that there is no conflict of interest.

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acta medica

ORIGINAL ARTICLE

Evaluation of Prognostic Markers in Cancer-associated Fibroblast Based Sub-groups of Colorectal Cancer

Seçil Demirkol Canlı
ORCID: 0000-0003-0200-7962

ABSTRACT

Objective: Stromal cells in the tumor microenvironment (TME) are among the main players of carcinogenesis. Cancer-associated fibroblasts (CAFs) residing in tumor stroma are involved in cancer progression through various mechanisms, supporting tumor growth, cellular motility and invasiveness. The discovery of markers predicting recurrence risk in colorectal cancer (CRC) have led to the generation of several gene panels, including Coloprint. This study aimed to understand the impact of a CAF-rich and a CAF-poor TME on the performance of the prognostic markers in Coloprint.

Materials and Methods: Publicly available transcriptomic data of CRC tumors were used to generate tumor sub-groups based on CAF specific gene expression. Subsequently, prognostic relationships of Coloprint genes were assessed within these subgroups.

Results and Conclusion: Our data revealed that prognostic performance of Coloprint genes differed dramatically between CAF stratified subgroups compared to non-stratified analysis. We have found that multiple genes lost their prognostic significance and several genes showed an association in the opposite direction. 9 out of 17 genes were differentially expressed in at least one of the CAF-specific subgroups and majority of the genes predicted prognosis independent of CAF levels. These findings showed that the performance of the prognostic markers can vary significantly among CAF-poor and CAF-rich groups. Therefore testing potential biomarkers within such biological sub-groups may contribute to the development of more specific gene panels.

Keywords: Colorectal cancer, Coloprint, prognosis, biomarker, fibroblast, cancer- associated fibroblast

Corresponding Author: Seçil Demirkol Canlı
Molecular Pathology Application and Research Center, Hacettepe University, Ankara, Turkey.
E-mail: secil.demirkol@hacettepe.edu.tr

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer type and cause of cancer related death in both men and women in the USA [1]. According to the statistics of the Health Ministry of the Turkish Republic, in Turkey colorectal cancer is the fourth and second most common cancer type in men and women, respectively [2]. The 5-year survival of CRC patients is 64%, ranging from 90% for localized disease to 14% for advanced-stage disease [3]. Clinical factors defining poor prognosis for CRC include obstruction and perforation of colon at diagnosis, low number of assessed lymph nodes (<12), T4 stage, high grade, vascular, lymphatic or perineural invasion and residual tumor [4]. It is well known that diseases at stages II and III can show varying clinical outcomes upon treatment, therefore gene expression based molecular tests have been also developed to assess patients at risk of recurrence or patients who are unlikely to benefit from conventional therapy. To address this issue, Salazar et al. evaluated microarray based expression profile of 188 CRC patients at various

stages (I to IV), and identified a set of 18 genes that were associated with metastasis-free survival [5]. This signature, called Coloprint, was then validated using an independent set of 206 samples from patients with stage I, II, and III CRC [5]. Multivariate analyses showed that Coloprint was a strong independent prognostic factor in both stage II and III diseases, and was superior to the ASCO criteria for the evaluation of recurrence risk in stage II patients (HR=3.34; p=0.017) [5].

The crosstalk between various cell types in TME has a major impact on tumor progression [6]. Numerous studies have shown that a sub-population of fibroblasts, called cancer-associated fibroblasts (CAFs) or 'activated fibroblasts' in the tumor stroma are prominent promoters of tumor growth and progression [7]. During tumor progression, fibroblasts are activated by *TGFβ*, monocyte chemotactic protein 1 (*CCL2*), and extracellular matrix (ECM) degrading agents such as matrix metalloproteinases (MMPs) [8]. The activated CAFs in turn affect cellular motility by secreting various growth factors and cytokines. They are also an important source of MMPs, which degrade and remodel ECM, thus enhancing tumor growth, invasion, angiogenesis, recruitment of inflammatory cells, and metastasis [7,9,10]. In CRC, elevated CAF signature has been associated with poor disease-free survival in patients who did not receive adjuvant chemotherapy [11]. A CAF index was found to be even more powerful than an epithelial-mesenchymal transition (EMT) score in predicting survival outcomes in a pan-cancer cohort [12]. In line with that, transcriptomic based fibroblast scores were higher in the consensus molecular subtype 4 (CMS4) of CRC tumors, which have the worst prognosis [13].

CAF-rich and CAF-poor tumors show differences in molecular dynamics, prognosis and aggressiveness, however the evaluation of prognostic markers has not been previously addressed in CAF level stratified tumors. The lack of this knowledge prompted us to re-evaluate a panel of prognostic markers in CRC tumors with different levels of CAFs. In order to do that, hierarchical clustering analysis based on expression of six CAF specific markers was performed in colorectal tumors. Then, a previously published and validated prognostic gene panel, Coloprint, was re-tested in stage II and III CRC separately in the aforementioned CAF based sub-groups. Majority of these markers showed loss of significance and significance in opposite directions in prognostic analyses when stratified by CAF levels. Overall, our results suggest that gene panels developed for risk prediction may lose their power if tumors are further subdivided into subgroups with different biological features.

MATERIALS AND METHODS

Study cohorts and microarray data processing

CEL files of colorectal tumors within GSE39582 [14], GSE17536 [15] and GSE14333 [16] datasets were downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) and RMA normalized using "affy" package in R Bioconductor [17]. Clinical data was obtained from Array Express (http://www.ebi.ac.uk/arrayexpress). Consensus molecular subtype information was downloaded from Synapse platform (www.synapse.org) for samples in GSE39582.

MCP-counter and ESTIMATE

MCP-counter R package was downloaded from Zenodo (https://doi.org/10.5281/zenodo.61372). Based on probeset level expression data as input, scores for fibroblasts were obtained separately for GSE39582, GSE17536 and GSE14333. Stromal scores were obtained via ESTIMATE R package (https://bioinformatics.mdanderson.org/estimate/rpackage.html) for GSE39582 [18]. Samples were sorted from lowest to highest based on stromal scores and divided into three groups including 188, 189 and 189 samples with low, intermediate and high score, respectively.

Hierarchical Clustering

Cluster 3.0 and Treeview programs were used for hierarchical clustering and visualization of heatmaps (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv). Gene expression data was standardized to mean of zero and standard deviation of 1 for each gene. This data was then used as input for Cluster 3.0 software. Hierarchical clustering was performed using euclidian distance as similarity metric and complete linkage as clustering method. The output in ".cdt" format was used as input for Treeview software for visualization and heatmap generation.

Survival Analyses

Univariate cox regression analysis was performed to evaluate prognostic relationships using continuous gene expression data. For genes in Coloprint with multiple probesets, one probeset was selected to be used throughout the study using the following criteria: 1. For each probeset log-rank tests were performed at all possible cut-offs within 10-90 percentiles using "survival" package in R Bioconductor 2. The lowest p value for each probeset was noted, which was named "best cut-off p value" 3. The probeset with the lowest "best cut-off p value" was selected and used for each gene.

For categorical analyses of survival, expression based cut-off with the lowest log-rank p value within 25-75 percentiles was preferred in order to define low and high expression groups. If there was no significance in any of the cut-offs in 25-75%, then the cut-off resulting in the lowest p value within 10-90% range was used. Log-rank p values smaller than 0.05 were considered significant. If no significant p value was obtained at any of the cut-offs tested within 10-90 percentiles, the gene was considered not significantly associated with prognosis. Patients with survival time "0" were excluded from all survival analyses.

Statistical Analyses

Gene expression plots comparing CAF groups were generated using "ggplot2" package [19], and ANOVA was performed using "oneway.test" function in R Bioconductor [20]. Pearson correlation analysis was performed using Microsoft Excel (2013) for the evaluation of intergenic correlations among CAF markers. Kaplan Meier graphs were generated using GraphPad Prism version 6 for Windows (Graphpad Software, San Diego, CA, USA) and cox regression analyses were performed using IBM SPSS Statistics for Windows, Version 23.0 (Armonk, NY, IBM Corp).

RESULTS

Relationship of gene expression and prognosis in stage II and III CRC

To assess the prognostic value of Coloprint genes in CAF-specific biological sub-groups, a transcriptomic based workflow was applied as summarized in Figure 1. Univariate cox regression analyses were performed for the genes included in the signature with recurrence-free survival (RFS) using microarray data from stage II and III CRC tumors in GSE39582 dataset (n=253 and n=200 with available recurrence-free survival data, respectively). 17 out of 18 genes in Coloprint were available in this dataset. Cox regression analyses showed that 7 and 2 genes were significantly associated with RFS in stage II and III patients, respectively (Table 1). However, expression values used in a continuous fashion did not show a significant prognostic relationship for the majority of the genes in both stage II and III disease (Table 1); therefore, the prognostic values were evaluated

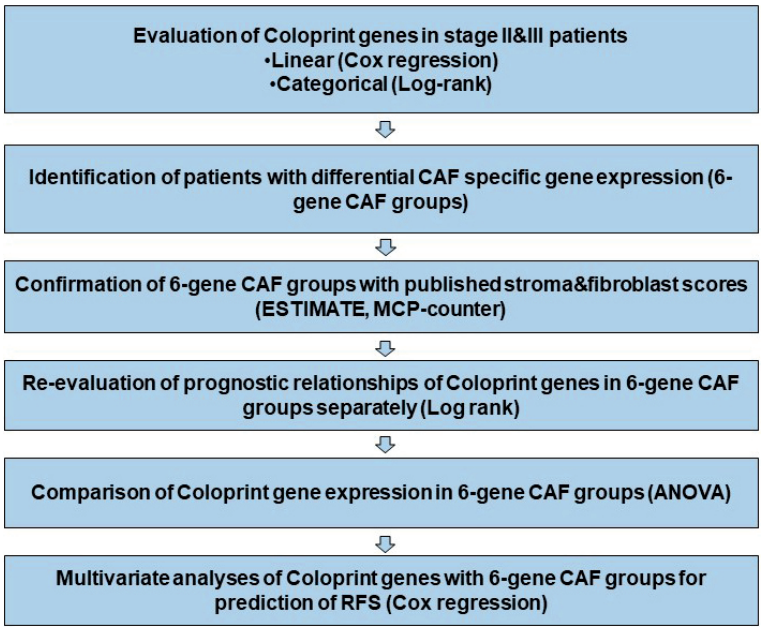

Figure 1. A schematic of workflow of the study.

Table 1. Univariate cox regression analyses of 17 genes in stage II & III tumors (GSE39582, RFS).

	Stage II				Stage III			
			95% CI for HR				95% CI for HR	
	p	HR	lower	upper	p	HR	lower	upper
CTSC	**<0.001**	1.932	1.35	2.766	0.449	0.877	0.625	1.231
CYFIP2	0.365	0.849	0.595	1.21	0.914	0.982	0.712	1.355
EDEM1	0.344	0.747	0.409	1.365	0.43	0.825	0.512	1.33
HSD3B1	0.079	3.376	0.868	13.127	0.131	2.556	0.757	8.63
IL2RA	**0.001**	2.126	1.386	3.261	0.202	0.76	0.498	1.159
IL2RB	**0.032**	1.439	1.032	2.006	**0.002**	0.58	0.407	0.825
LAMA3	0.083	2.49	0.888	6.982	0.128	1.862	0.835	4.151
LIF	**0.012**	1.596	1.11	2.295	0.594	1.09	0.793	1.498
MCTP1	0.211	0.82	0.6	1.12	0.377	0.88	0.662	1.169
PIM3	0.401	1.288	0.713	2.327	0.816	1.064	0.632	1.789
PLIN3	0.9	0.972	0.62	1.522	0.675	1.091	0.726	1.638
PPARA	**0.001**	0.515	0.352	0.754	0.356	0.837	0.574	1.221
PYROXD1	**0.011**	0.755	0.609	0.937	0.063	0.786	0.611	1.013
SLC6A11	0.072	2.313	0.927	5.774	**0.044**	2.137	1.021	4.475
THNSL2	**0.016**	1.693	1.105	2.592	0.489	0.883	0.622	1.255
ZBED4	0.1	0.664	0.408	1.081	0.184	0.778	0.537	1.127
ZNF697	0.888	0.914	0.258	3.232	0.557	1.313	0.53	3.255

using expression data categorically.

When the prognostic relationship of each gene was re-evaluated by comparing prognosis of the tumor groups with high and low expression (see Methods) for stage II and stage III patients separately, a significant relationship with RFS was observed for 13 genes in stage II and 12 genes in stage III disease (Table 2). In a pooled analysis of 453 stage II and III patients, high expression of 7 genes (*CTSC, HSD3B1, IL2RA, LAMA3, LIF, SLC6A11, THNSL2*) and 6 genes (*CYFIP2, EDEM1, MCTP1, PPARA, PYROXD1, ZBED4*) were associated with shorter and longer RFS, respectively (Table 2). Significant relationships in the opposite directions were noted for *CTSC, IL2RA, IL2RB* and *THNSL2* genes. The expression of these genes were associated with shorter RFS in either stage II or pooled analyses, while they were associated with longer RFS in stage III disease (Table 2). In brief, multiple Coloprint genes did not show consistent prognostic associations in stage II and III patients in GSE39582.

Sub-grouping method based on CAF specific gene expression

To define sub-groups based on CAF specific gene expression, six known CAF markers were used, *ATL1* [21], *PDGFRA, PDGFRB, FAP, ACTA2, S100A4* [22]. Based on intergenic correlations of all probesets of 6 CAF markers in 566 colorectal tumors from GSE39582 dataset (Table 3), the probeset with the highest mean Pearson r value was used in further analyses for genes with multiple probesets. Hierarchical clustering analyses performed separately for tumors in GSE39582 (n=566), GSE17536 (n=177) and GSE14333 (n=290) datasets showed 3 sub-groups with clear high, intermediate and low expression of markers consistently in 3 datasets (Figure 2, Supplementary Table 1). This classification was named "6-gene CAF groups" and used accordingly throughout the study. In line with these findings, mean expression of the six markers was significantly different among groups (Supplementary Figure 1).

In order to confirm that the groups represent enrichment of CAFs in the tumor microenvironment, a previously published algorithm called "MCP-counter" was used. This algorithm predicts the abundance of various cell types in the tumor microenvironment based on transcriptomic profiles, including fibroblasts [23]. Based on the MCP-counter algorithm, tumors were significantly infiltrated by fibroblasts ($p<0.05$) in GSE39582 (100% of all samples), GSE17536 (100% of all samples), and GSE14333 (99.3% of all samples) datasets. Among 6-gene CAF groups, CAF high

Table 2. Univariate cox regression analyses of 17 genes in stage II & III tumors (GSE39582, RFS).

Probeset	Gene	Stage II&III (n=453)		Stage II (n=253)		Stage III (n=200)	
		HR	P	HR	P	HR	P
225646_at	CTSC	1.5394	0.0118	2.5089	0.0006	0.5446	0.0118
215785_s_at	CYFIP2	0.4712	0.0255	ng	ns	ng	ns
203279_at	EDEM1	0.5615	0.0281	ng	ns	0.4707	0.0388
241111_at	HSD3B1	1.7473	0.0054	1.9253	0.0138	1.6385	0.0256
211269_s_at	IL2RA	1.6919	0.0355	2.8776	0.0005	0.2973	0.0117
205291_at	IL2RB	ng	ns	2.2127	0.0194	0.4095	0.0000
1568879_a_at	LAMA3	1.9139	0.0002	1.9588	0.0147	1.9082	0.0047
205266_at	LIF	2.0601	0.0009	2.3729	0.0022	1.6982	0.0264
235740_at	MCTP1	0.6115	0.0299	0.3025	0.0326	ng	ns
224739_at	PIM3	ng	ns	2.0521	0.0176	ng	ns
202122_s_at	PLIN3	ng	ns	ng	ns	ng	ns
226978_at	PPARA	0.4146	0.0000	0.3833	0.0002	0.5071	0.0225
213878_at	PYROXD1	0.4996	0.0000	0.3708	0.0001	0.5492	0.0076
230286_at	SLC6A11	2.3649	0.0002	1.9061	0.0167	1.9048	0.0068
219044_at	THNSL2	1.6922	0.0222	1.8598	0.0187	0.6014	0.0304
204799_at	ZBED4	0.5282	0.0011	0.5673	0.0483	0.6128	0.0414
1553702_at	ZNF697	ng	ns	ng	ns	ng	ns

ns: not significant
ng: HR was not given for nonsignificant relationships
Yellow and blue colors indicate relationships with poor (HR>1) and good (HR<1) prognosis, respectivelly.

Table 3. Intergenic correlation of CAF marker expression to select representative probesets (GSE39582, n=566).

		ACTA2	ACTA2	ACTA2	ATL1	FAP	PDGFRA	PDGFRA	PDGFRA	PDGFRA	PDGFRB	S100A4
		200974_at*	215787_at	243140_at	**223340_at***	**209955_s_at***	**203131_at***	211533_at	215305_at	1554828_at	**202273_at***	**203186_s_at***
ACTA2	**200974_at**	1.000	0.058	0.567	0.380	0.634	0.628	-0.090	0.284	0.167	0.806	0.482
ACTA2	215787_at	0.058	1.000	0.174	-0.014	0.012	0.015	-0.009	0.076	0.137	0.026	-0.036
ACTA2	243140_at	0.567	0.174	1.000	0.209	0.417	0.373	-0.036	0.204	0.161	0.486	0.294
ATL1	**223340_at**	0.380	-0.014	0.209	1.000	0.267	0.414	-0.093	-0.099	-0.061	0.178	0.257
FAP	**209955_s_at**	0.634	0.012	0.417	0.267	1.000	0.419	-0.157	0.225	0.030	0.700	0.493
PDGFRA	**203131_at**	0.628	0.015	0.373	0.414	0.419	1.000	-0.127	0.337	0.045	0.569	0.249
PDGFRA	211533_at	-0.090	-0.009	-0.036	-0.093	-0.157	-0.127	1.000	-0.077	0.163	-0.095	-0.037
PDGFRA	215305_at	0.284	0.076	0.204	-0.099	0.225	0.337	-0.077	1.000	0.140	0.389	0.187
PDGFRA	1554828_at	0.167	0.137	0.161	-0.061	0.030	0.045	0.163	0.140	1.000	0.165	0.132
PDGFRB	**202273_at**	0.806	0.026	0.486	0.178	0.700	0.569	-0.095	0.389	0.165	1.000	0.491
S100A4	**203186_s_at**	0.482	-0.036	0.294	0.257	0.493	0.249	-0.037	0.187	0.132	0.491	1.000
	Mean r †	0.447	0.131	0.350	0.222	0.367	0.357	0.040	0.242	0.189	0.429	0.319

r values are colored in red, white and green from highest to lowest, respectively
*Probesets used in further analyses. Probeset with the highest mean r was selected for genes with multiple probesets.
†Columnwise average r value

group had the highest MCP-counter fibroblast score and the score gradually and significantly decreased in intermediate and low groups (Supplementary Figure 2). Therefore, MCP-counter fibroblast scores were highly consistent with the 6-gene CAF groups. As seen in Table 4, tumor groups defined by another method, ESTIMATE algorithm, designed to predict presence of infiltrating stromal cells [18], overlaps to a large extent with 6-gene CAF groups. 91.3% of CAF high tumors had high stroma and 89.8% of CAF low tumors had low stroma scores by ESTIMATE algorithm. These findings suggest that the 6-gene CAF groups identified in the current study were in line with tumor sub-groups defined by previously published CAF and stromal scoring methods and can clearly enable stratification of CRC tumors based on the level of CAFs in the tumor microenvironment. The 6-gene CAF groups also stratified patients with significantly different RFS, when evaluated in a pooled analysis of all stages and in stage II & III disease (Figure 3).

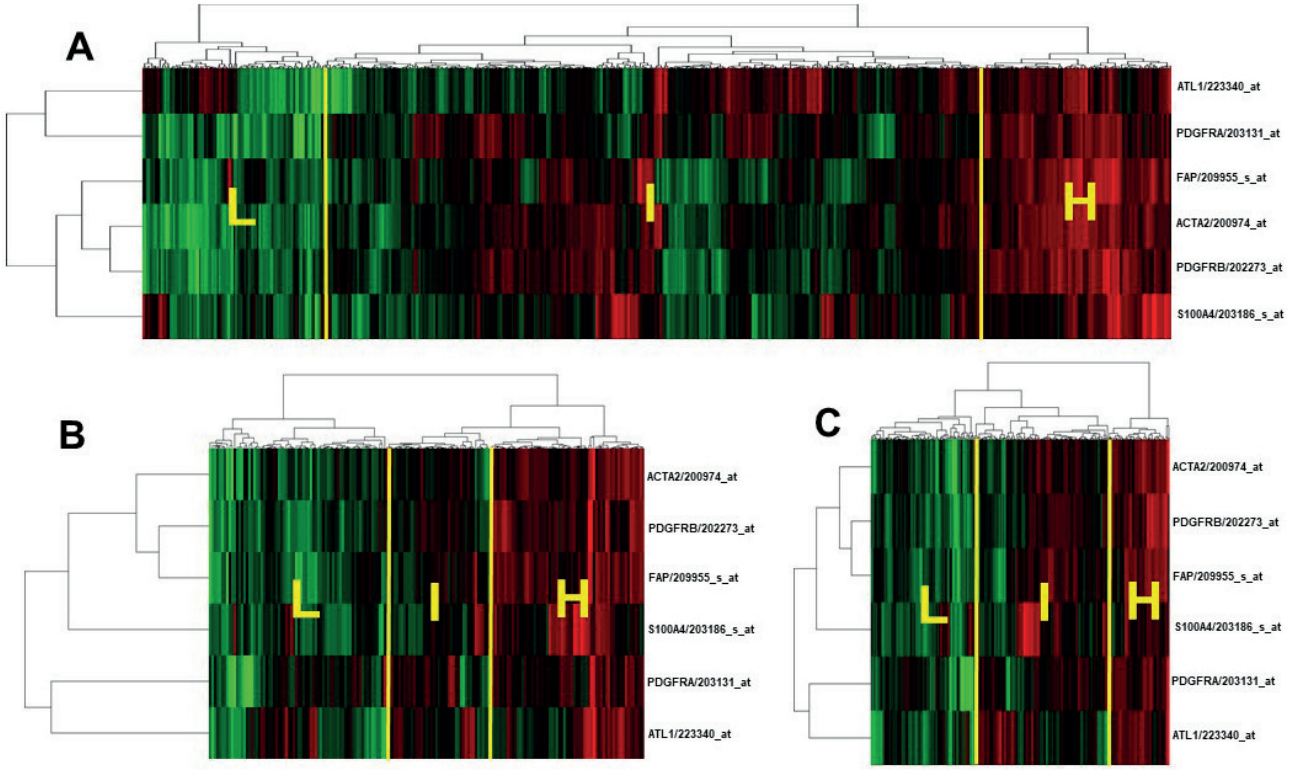

Figure 2. Hierarchical clustering analyses of colorectal tumors based on CAF markers. 566, 177 and 290 tumors were included in GSE39582 (A), GSE17536 (B), GSE14333 (C), respectively. «L», «I» and «H» letters indicate groups with low, intermediate and high CAF marker expression.

Table 4. Distribution of ESTIMATE (stroma) and 6-gene CAF groups (GSE39582, n=566).

		6 gene CAF groups			TOTAL
		LOW	INT	HIGH	
ESTIMATE groups	LOW	88 (89.8%)	100 (27.4%)	0 (0%)	188 (33.2%)
	INT	9 (9.2%)	171 (46.8%)	9 (8.7%)	189 (33.4%)
	HIGH	1 (1%)	94 (25.8%)	94 (91.3%)	189 (33.4%)
	TOTAL	98 (100%)	305 (100%)	103 (100%)	566 (100%)

Analysis of the distribution of CMS subtypes in CAF groups showed that 83.3% of the CAF high samples were CMS4 type (Supplementary Table 2), which was characterized as the mesenchymal subtype harboring prominent *TGFβ* activation, stromal invasion and angiogenesis [24]. The CAF intermediate group was heterogeneous and consisted of 20.2%, 54.7%, 12.4%, and 12.7% of the samples in CMS1, CMS2, CMS3 and CMS4 types, respectively. The CAF low group included only one CMS4 patient and the rest of the samples were distributed as 24.2% CMS1, 44.2% CMS2, and 30.5% CMS3. Overall, the CAF intermediate and low groups showed a heterogeneous distribution of CMS types, whereas CAF high group highly overlapped with tumors of CMS4 phenotype.

Evaluation of prognostic genes in 6-gene CAF groups

To re-assess the prognostic value of Coloprint genes within each 6-gene CAF groups, log-rank tests were performed based on the expression of each gene in a categorical fashion (see Methods) in stage II and III patients. As individual evaluation of prognostic relationships in each sub-group would reduce the sample sizes dramatically, this analysis was restricted to GSE39582 dataset, which has the highest number of samples with available survival data. Only three genes, *PPARA*, *PYROXD1* and *SLC6A11*, were significantly associated with RFS in all 6-gene CAF groups (Table 5). Hazard ratios (HR) indicated that high expressions of *PYROXD1* and *PPARA* were associated with longer RFS, and high expression of *SLC6A11* was associated with shorter RFS in all groups tested. Three genes, *CTSC*, *CYFIP*2 and *ZNF697* were significant markers of RFS

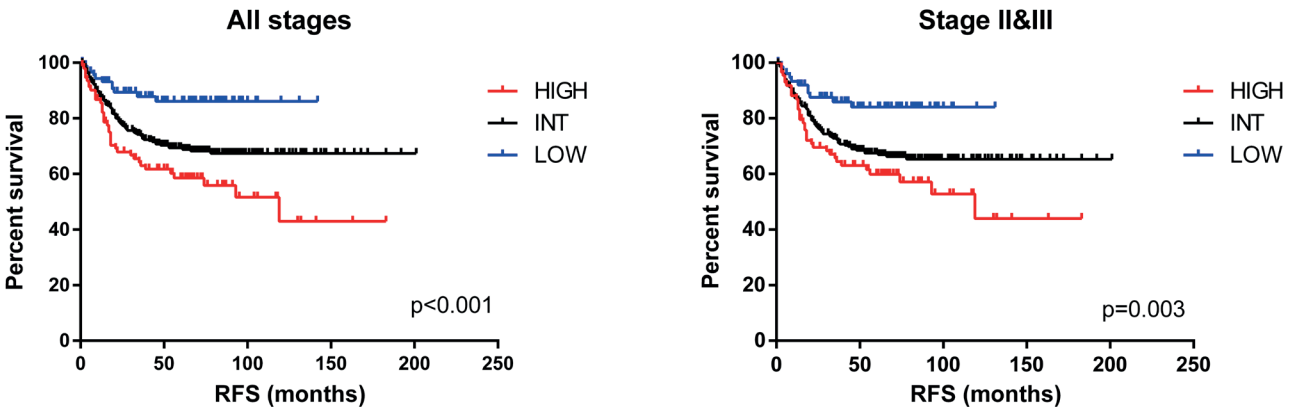

Figure 3. 6 gene CAF groups can predict RFS in GSE39582. Low, intermediate (INT) and high groups were assigned as defined in Figure 1. Log-rank p values are indicated.

in only the CAF high group. *EDEM1, MCTP1* genes were significantly related to longer RFS and high *THNSL2* expression was related to shorter RFS in CAF low and CAF intermediate groups, but not in CAF high, suggesting that high CAF content of the microenvironment might interfere with the prognostic role of these genes. The genes *ILR2A, LIF* and *ZBED4* had prognostic value in CAF low and CAF high groups, but not in CAF intermediate group, whereas *LAMA3* was significant in CAF intermediate and CAF high groups. *PIM3* was associated with unfavorable RFS in only CAF low group. *PLIN3* was the only gene that is not significantly related to prognosis in any of the groups and in the pooled analyses. These data overall indicate that the prognostic relationships of most of these validated markers were highly heterogeneous when the tumors were stratified by CAF levels.

Interestingly, two genes, *HSD3B1* and *IL2RB* had contradictory relationships when evaluated in the 6-gene CAF groups separately. *HSNDB1* expression was significantly related to poor RFS in pooled analyses and the CAF intermediate group, whereas it was associated with good RFS in the CAF high group (Table 2&5). *IL2RB* was related to shorter RFS

Table 5. Log-rank based analyses of prognosis in 6-gene CAF groups (GSE39582, stage II&III, RFS).

Gene	Low (n=77)		Int (n=290)		High (n=86)	
	HR	P	HR	P	HR	P
CTSC	ng	ns	ng	ns	3.9437	0.0140
CYFIP2	ng	ns	ng	ns	0.2539	0.0003
EDEM1	0.1878	0.0169	0.574659	0.047502	ng	ns
HSD3B1	ng	ns	1.6814	0.0189	0.3673	0.0034
IL2RA	6.7471	0.0352	ng	ns	2.2592	0.0491
IL2RB	3.3433	0.0416	0.5384	0.0040	ng	ns
LAMA3	ng	ns	2.2358	0.0001	2.3384	0.0133
LIF	5.4958	0.0024	ng	ns	2.5422	0.0193
MCTP1	na*	0.0374	0.626602	0.025783	ng	ns
PIM3	3.4145	0.0328	ng	ns	ng	ns
PLIN3	ng	ns	ng	ns	ng	ns
PPARA	0.3077	0.0409	0.5658	0.0475	0.4039	0.0123
PYROXD1	0.2821	0.0330	0.5876	0.0131	0.3738	0.0028
SLC6A11	3.7744	0.0193	2.0680	0.0106	4.3548	0.0078
THNSL2	13.0820	0.0015	1.6135	0.0299	ng	ns
ZBED4	0.1639	0.0011	ng	ns	0.4421	0.0168
ZNF697	ng	ns	ng	ns	0.4241	0.0312

*Not available. Cox model resulted in an unrealistic HR due to lack of event in one of the groups
ns: not significant
ng: HR was not given for nonsignificant relationships
Yellow and blue colors indicate relationships with poor (HR>1) and good (HR<1) prognosis, respectivelly.

in CAF low, but longer RFS in the CAF intermediate group (Table 5). This type of opposite pattern was also observed in stage stratified analyses. High expression of *IL2RB* was associated with bad and good prognosis in stage II and stage III, respectively (Table 2). These findings were quite striking, since the prognostic relationships could show significant, but opposite patterns within 6-gene CAF groups, suggesting that CAF related changes in tumor microenvironment may have an effect on gene expression based prognostic predictions.

When expression of these genes between CAF groups were compared in stage II & III patients, 9 out of 17 genes (*CTSC, PPARA, ZBED4, MCTP1, IL2RA, IL2RB, PYROXD1, PIM3, SLC6A11*) showed significantly different expression in at least one CAF group (ANOVA $p<0.05$). Mean expression of *PPARA, ZBED4, PYROXD1, PIM3* decreased while mean expression of *CTSC, MCTP1, IL2RA, IL2RB, SLC6A11* increased gradually with increasing CAF level (Figure 4). Next, multivariate cox regression analyses (MVA) of 6-gene CAF groups were performed with each of the 13 genes which were significantly related to prognosis in a pooled analyses of stage II and III patients (Table 2). Our results indicated that 10 genes (*EDEM1, HSD3B1, LAMA3, LIF, MCTP1, PPARA, PYROXD1, SLC6A11, THNSL2, ZBED4*) were related to RFS independent of 6-gene CAF groups while 3 genes (*CTSC, CYFIP2, IL2RA*) were not (Supplementary Table 3). *CTSC* and *IL2RA* genes were the only two genes, expression of which were elevated with increasing CAF levels and which lost significance in the multivariate cox model, indicating that the prognostic groups identified by these genes and 6-gene CAF groups were highly overlapping.

DISCUSSION

Although it is known that CAFs are involved in tumor progression through secretion of various oncogenic signals and ECM-degrading proteases in the tumor microenvironment [25], how CAF involvement might affect the performance of putative prognostic or predictive biomarkers has not been elucidated so far. In this study, publicly available microarray data of CRC tumors were utilized and tumor sub-groups with low, intermediate and high CAF marker expressions were generated. This method enabled the identification of clear CAF sub-groups in three independent microarray datasets and these CAF groups were then confirmed with two previously published scoring methods for fibroblasts and tumor stroma. Therefore it is a fast, robust and practical way of obtaining CAF

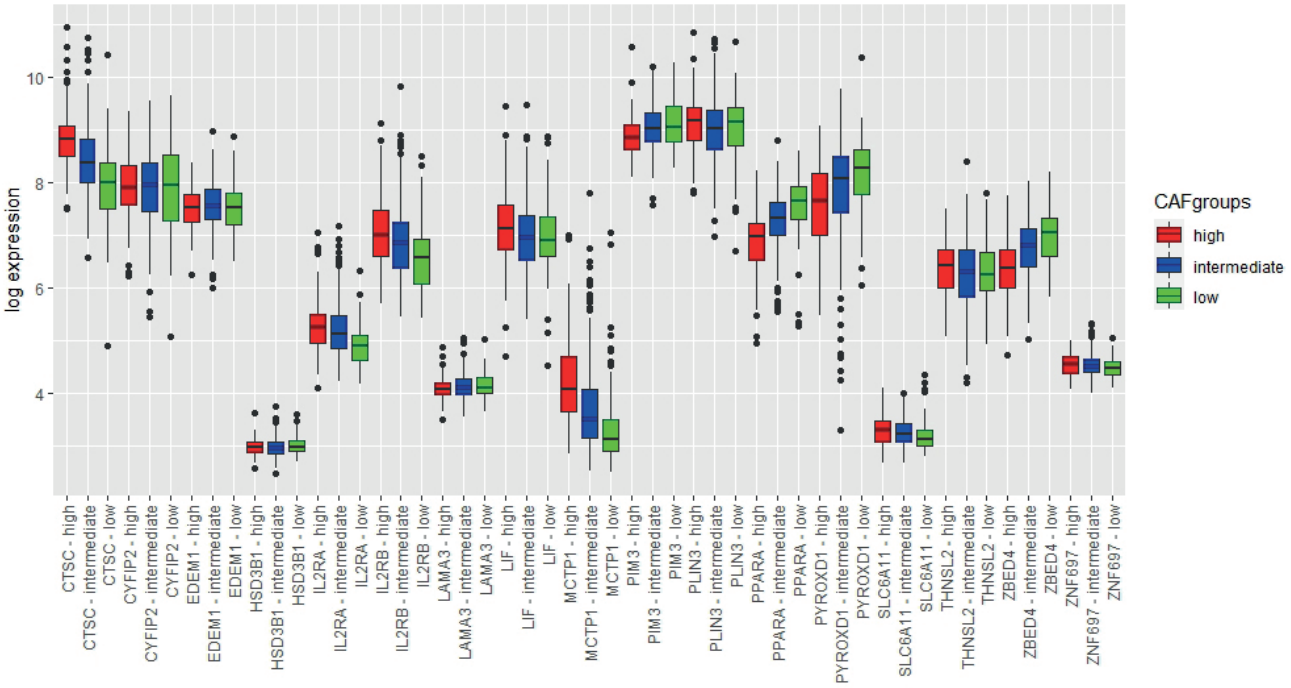

Figure 4. Expression of Coloprint genes in 6-gene CAF groups. Boxes extend from 25th to 75th percentiles. The upper and lower whiskers extend 1.5 times the interquartile range above the upper quartile and below the lower quartile (Q1 - 1.5 * IQR or Q3 + 1.5 * IQR). Data beyond the end of the whiskers, outliers, are plotted individually.

related sub-groups based on transcriptomic data of CRC tumors. The significant gradual increase observed in mean expression of the 6 markers in 6-gene CAF groups, suggests that calculating mean expression of these markers could be noted as a practical and alternative approach to generate similar sub-groups. Our classification method may be also useful for transcriptomic data obtained by other technologies, such as RNA-seq and qRT-PCR, however further studies are needed to confirm the applicability.

Evaluation of gene expression-prognosis relationships showed that the biomarkers exhibit a heterogeneous pattern of significance in CAF groups. Only 3 genes *PPARA, PYROXD1* and *SLC6A11* out of 17 were significantly associated with clinical outcome in all 6-gene CAF groups. Furthermore, two genes *HSD3B1* and *IL1R3B* were significantly associated with RFS in the opposite directions in different CAF groups. The data further showed that CAFs are indeed a significant contributor to consensus molecular subtypes of colorectal cancer, as 83.3% of CAF high tumors were CMS4 type which is associated with an EMT phenotype and activation of matrix remodeling, angiogenesis and a gene expression profile compatible with stromal infiltration [24]. It is also known that CMS4 tumors have active *TGFβ* signaling [24], further supporting a CAF-rich microenvironment since *TGFβ*, released by cancer cells, is one of the key mediators of fibroblast activation [8]. Overall, these findings suggest that CAF levels may or should be considered as an important factor while evaluating putative markers as they contribute to significant changes in the tumor microenvironment that may affect the performance of biomarkers in prognostic panels.

In this study, the prognostic relationships were analyzed in a categorical way, via comparing high/low expression groups at all possible cut-offs. This approach enabled the identification of relationships that were weak to be significant in an analyses applied with the continuous log expression values. GSE39582 dataset was used for assessment of prognostic relationships in each 6-gene CAF group. There were 90, 336 and 93 patients with nonzero RFS and status information in CAF low, intermediate and high groups respectively. Therefore high sample size in this dataset enabled further dividing each 6-gene CAF group into two groups based on expression of individual genes for prognostic comparison. Although the expression patterns of the six CAF markers were confirmed in two other independent datasets (GSE17536, GSE14333), these datasets were not utilized for prognostic analyses within CAF groups. GSE14333 had 44 samples in CAF high group with available clinical outcome data, and GSE17536 included 38 in CAF intermediate group. These sample sizes can be considered relatively low for prognostic comparisons, as categorical evaluations based on gene expression in these groups will lead to the comparison of data from only 15-20 patients to others. Thus, the categorical prognostic evaluations within each 6-gene CAF group were not performed in these datasets.

Upon evaluation of prognostic relationships of Coloprint genes, we noted clear changes in HR and p values for multiple genes when analyzed separately in 6-gene CAF groups. As Coloprint was developed based on Agilent oligonucleotide arrays [5], the platform-based differences such as the hybridization of probes to different transcript variants might have altered the direction and significance of prognostic relationships. In addition, differences in cohort-specific clinical characteristics may have had an effect on these inconsistencies.

The molecular function of genes in Coloprint included roles in cell proliferation, immune response, metabolism and cell invasion [5]. Among the genes involved in this signature, several genes have been previously linked to CAFs and CAF related molecular mechanisms. Laminin-332, an extracellular matrix (ECM) component composed of *LAMA3, LAMB3*, and *LAMC2* chains, was highly expressed in the tumor-normal interface. It was suggested that this may be a product of a paracrine intercellular reaction between invasive tumor cells in the tumor core and myofibroblasts in the tumor-normal interface to provide a suitable microenvironment for invasion in breast cancer [26]. Therefore, the fact that *LAMA3* was a significant predictor of prognosis in CAF high and CAF intermediate groups but not CAF low group, further supports that the role of this gene in prognostic prediction may rely on the presence of myofibroblasts in the microenvironment. Expression of *CTSC*, which positively correlated with CAF levels in our study, is expressed by fibroblasts and immune cells that mediates angiogenesis and growth of transplantable tumors [27]. In the current

study, *CTSC* was associated with RFS in only the CAF high group suggesting that *CTSC* expressed by CAFs may be relevant to its prognostic role. This is also in line with its lack of significance in a cox model including CAF levels in MVA. The expression of Leukemia inhibitory factor (*LIF*), which is secreted by both fibroblasts and neoplastic cells, is triggered by *TGFβ*. *LIF* is also involved in pro-invasive activation of stromal fibroblasts [28]. Although not significant, *LIF* expression was relatively higher in CAF high group, therefore it's likely that its role in the activation of fibroblasts might contribute to its prognostic associations.

Contradictory results in the direction and significance of multiple prognostic relationships upon individual evaluation of CAF groups, suggests that other prognostic markers or gene panels proposed in the literature may also show divergence in performance in CAF rich and CAF poor tumor microenvironments. Therefore it would be useful to take the involvement of CAFs into account while evaluating potential biomarkers and tumor sub-grouping gene panels.

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Author contribution

Study conception and design: SDC; data collection: SDC; analysis and interpretation of results: SDC; draft manuscript preparation: SDC. The author reviewed the results and approved the final version of the manuscript.

Ethical approval

All data used in this study is from public databases as described in the methods, for which no ethical approval is required.

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Conflict of interest

The author declares that there is no conflict of interest.

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acta medica

ORIGINAL ARTICLE

From Thesis to Publication - Analysis of 2580 theses in the field of Anesthesiology and Reanimation

İlke Küpeli[1]
ORCID: 0000-0003-3518-7365

Merve Yazıcı Kara[2]
ORCID: 0000-0003-4902-4428

Ayşe Kayhan[2]
ORCID: 0000-0002-6856-8060

[1]Department of Anesthesiology and Reanimation, Biruni University Faculty of Medicine, Istanbul, Turkey.

[2]Department of Anesthesiology and Reanimation, Derince Training and Research Hospital, Kocaeli, Turkey.

Corresponding Author: İlke Küpeli
Department of Anesthesiology and Reanimation, Biruni University Faculty of Medicine, Istanbul, Turkey.
E-mail: ilkeser2004@gmail.com

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ABSTRACT

Objectives: The primary aim of the study was to determine the publication rates of the theses produced in the department of Anesthesiology and Reanimation in Turkey between the years 2000 and 2018.

Methods: This cross-sectional study evaluated 2580 theses identified in the National Thesis Center using the keywords "Anesthesiology and Reanimation", to which online access was granted by the authors. Theses that had restricted access or were accepted as abstracts in congresses were excluded. The gender of the thesis writers, the date of completion of the thesis and the year of publication, the place where the thesis research was conducted (university or ministry of health), the field of thesis subject, the database where the publication is located, and whether the author began an academic career after the thesis, was investigated.

Results: Thesis authors were female in 52.4% of the 2580 theses included in the study while 59.1% were in the field of general anesthesia and 14.4% in the intensive care field. The proportion of authors obtaining an academic career was 15.5%. The rate of publication was 39.1% (n=1010). The mean time to publication was 3.46±2.62 years. Authors who established an academic career were significantly more likely to publish (72.5%; p<0.001)

Conclusion: Although higher than previously reported, thesis publication rate remains low at 39% although a higher proportion of authors were female. Furthermore, the time to publication was long.

Keywords: Publication rates, theses, academic careers, Anesthesiology and Reanimation

INTRODUCTION

Postgraduate education is a process that aims to educate scientists, who can solve problems with a way of thinking that produces, uses, and criticizes knowledge. The planning and effective execution of postgraduate education is closely related to the level of development of the specific country in which the postgraduate education is delivered [1].

A part of this challenging process is the writing of the thesis. The primary goal of writing a thesis is to provide the candidate with experience of scientific study systematics, improve the ability to write articles, and generate new knowledge. It is expected that the research assistant, who prepares the thesis, should be able to form a hypothesis, be familiar with good clinical practices, collect data in a systematic and disciplined fashion, analyze the data appropriately, scan the literature, and interpret the results in the light of relevant literature [1]. The next step is to share the results of the study with the scientific community.

Unfortunately, thesis-publication rates are poor, both in Turkey and in other countries [2]. Research in Turkey has shown that thesis publication rates are very variable. In psychiatry publication rates have been reported to be 37.4% [3]. However in other specialities these rates tend to be much lower: 0.9% in family medicine; 7.3% in emergency medicine; 1.5% in public health; 4.2% in microbiology; 5.7% in urology; 6.8% in neurosurgery; 5% in general surgery; 3.8% in eye disease and surgery; 4.2% in ear, nose and throat disease and surgery. The overall publication rate of theses among all medical specialities was 6.6% in universities and 1.3% in public hospitals [4]. In a study conducted by Yılmaz et al. [5] in the field of Anesthesiology and Reanimation, the rate of publication of theses (n=1207) written by university hospital specialists was 11.3%.

The primary aim of this study was to determine the publication rates of theses published by both universities and the Ministry of Health in Turkey originating from Departments of Anesthesiology and Reanimation and to examine the characteristics of the publications.

MATERIALS AND METHODS

This study was planned as a cross-sectional study. By using the keywords "Anesthesiology and Reanimation" in the area of the main discipline on the website of the National Thesis Center of the Council of Higher Education (CHE), we identified 3210 medical specialty theses submitted between 2000 and 2018. Of these 2580 (80.4%) theses whose full data was accessible and to which we were given permission for online access by the authors were evaluated. The clinical research ethics committee of Kocaeli Derince Education and Research Hospital waived ethical approval for this study, since the theses authors had given permission for access of evaluated theses (ClinicalTrials.gov ID: NCT04663984). The study started on 25 December 2020 and was completed on 20 February 2021.

The theses of authors who died before publication and those theses that the authors declined online access to were excluded from the study. Furthermore, theses that had only been published as congress abstracts were not accepted to have not been fully published.

Publications, derived from theses, were extracted from the publishing databases Web of Science (WOS), SCI (Science Citation Index), SCI-E (Science Citation Index-Expanded, Extended Science Citation Index), Google, Google Scholar, and PubMed using authors, advisors name, and keywords.

Parameters collected included the gender of the thesis writers, the date of completion of the thesis and year of publication, the place where the thesis research was conducted (university/ministry of health), the field of thesis subject (general anesthesia, intensive care, algology, peripheral nerve block, neuraxial blocks), whether the thesis had been subsequently published, the delay between the date of the dissertation and the date of publication, the database where the publication was located (SCI/SCI-E; ULAKBIM-TRİNDEX; other international index; Index Not Scanned), the number of names of the thesis student and whether the author began an academic career after the thesis.

The primary aim of the study was to be able to determine the publication rates of the theses produced in the speciality Anesthesiology and Reanimation between the years 2000 and 2018.

The secondary purpose was to investigate factors affecting publication status, such as gender, university or health ministry education, the subject of the thesis, the writing status in the publication, the intention of pursuing an academic career.

Statistical analysis

Categorical variables are presented as numbers and percentages while continuous variables are presented as mean ± standard deviation and/or median and minimum–maximum. Comparison of the categorical variables between groups was done using Chi-square or Fisher exact test. The normality of distribution for continuous variables was investigated with the Kolmogorov–Smirnov test. The statistical level of significance for all tests was considered to be 0.05. Statistical analysis was performed using SPSS, version (IBM Inc., Armonk, New York, United States).

RESULTS

Thesis authors were female in 52.4% of the 2580 theses included in the study while 59.1% were in the field of general anesthesia and 14.4% in the intensive care field. Furthermore, 88.5% of the thesis authors were from university hospitals. Regardless of publication status, the rate of following an academic career was 15.5%. The rate of publication was 39.1% with 1010 thesis published (Table 1).

The characteristics of the published thesis were as follows. The average delay to publication was 3.46±2.62 years with a wide range from the same year to 19 years. Of the 1010 theses published, 61.4% were in the field of general anesthesia (p<0.001) and 51.9% were written by women. In addition, publication occurred in journals listed on SCI or SCI-E indexes in 36.7%. However, theses were published at a similar rate (36.3%) in other international indexed journals. The rate of the thesis author being the first author in the resulting publication was 89.6%. (Table 2). In addition, although the rate of publication from universities tended to be was higher than from the ministry of health, the difference was not significant (39.5% vs. 36.5%, respectively; p=0.343). The thesis publication rate of those who went on to have an academic career was 72.5% (p<0.001) compared to authors who did not have an academic career (Table 2).

Table 1. Demographic data

Variables	Subgroups	n=2580 (%)
Gender	Female	1352 (52.4)
	Male	1228 (47.6)
Area of the Thesis	General Anesthesia	1526 (59.1)
	Neuroaxial blocks	338 (13.1)
	Intensive Care	372 (14.4)
	Peripheral nerve blocks	244 (9.5)
	Algology	100 (3.9)
Institution	University	2284 (88.5)
	Ministry Of Health	296 (11.5)
Academic Career	Yes	401 (15.5)
Published	Yes	1010 (39.1)

Table 2. Data of Published Theses

Variables		n (%)	
Publication		1010 (39.1)	
Mean±SD (range) publication delay (years)	3.46 ± 2.62 (0-19)		
			p value
Area of the Thesis	General Anesthesia	621 (61.4)	<0.001*
	Neuroaxial blocks	152 (15.0)	
	Intensive Care	126 (12.4)	
	Peripheral nerve blocks	87 (8.6)	
	Algology	24 (2.6)	
Gender	Female	524 (51.9)	0.686
	Male	486 (48.1)	
Published Journal Index	SCI / SCI-E	371 (36.7)	<0.001**
	ULAKBIM (TR INDEX)	236 (23.3)	
	Other International	365 (36.3)	
	Non-Indexed	38 (3.7)	
Authorship Order	1	905 (89.6)	<0.001***
	2	83 (8.2)	
	3	15 (1.5)	
	4	5 (0.5)	
	5	2 (0.2)	
Institution[1]:	University	902 (39.5)	0.343
	Ministry of Health	108 (36.5)	
Academic career[1]	Yes	291 (72.5)	<0.001*
	No	719 (33.4)	

Chi-square, *general anesthesia vs others, ** SCI/SCI-E vs non-indexed, *** first author position vs others.

[1]Percentage of published 2580 theses scanned.

Table 3. Characteristics of The Published Thesis

Variables				n (%)
Publication		Yes		1010 (39.1)
		Institution, n (%)		
		University (902)	Ministry of Health (108)	
Academic career	No	656 (72.8)	63 (58,4)	0.002*
	Yes	246 (27.2)	45 (41,6)	
		Author position		
		1st author	2nd author	
Gender	Female	475 (52.5)	41 (49.4)	0.521
	Male	430 (47.5)	42 (50.6)	
Index	SCI / SCI-E	307 (33.9)	53 (63.8)	<0.001**
	ULAKBIM (TR INDEX)	221 (24.4)	9 (10.8)	
	Other international	343 (37.9)	19 (22.8)	
	Non-indexed	34 (37.5)	2 (2.4)	

*Chi-square, ** for 2nd author: SCI / SCI-E vs others

Characteristics of the theses are given in Table 3. Of authors who published their theses as an academic article, there was no gender difference among those who made an academic career (30.8% vs. 26.9%; p=0.18). The indexes in which the journals that published theses articles were unchanged by whether or not the authors had an academic career (p=0.089) or by their gender (p=0.84).

In addition, in the group who published their thesis, those who received their specialization from the Ministry of Health were more likely to pursue an academic career than those who received their specialization from a university (41.6% vs. 27.2%; p=0.002). There was also a relationship between the index listing the published journals and the author name order. (Table 3).

DISCUSSION

This study investigated the publication rate and characteristics of theses in the Anesthesiology and Reanimation speciality, written between 2000 and 2018 in Turkey. The publication rate was 39.1% and the mean delay between thesis acceptance and journal publication was 3.46±2.62 years. Furthermore, there was no difference in publishing rate when examined in terms of gender of the author or in university-based or health ministry-based authors.

Thesis writing plays a key role in the completion of postgraduate education in both Turkey and the rest of the world. Thesis writing assists the candidate to develop a scientific spirit as well as develop the ability to use inquiry and research techniques, and in the long term, provides analytical problem solving and the ability to critically interpret scientific writing. Considering that the research skills, analytical and organizational skills learned in writing a thesis will serve the candidates for a lifetime, the significance of writing and publishing a thesis-based article is high. However, the publication rate of medical theses is much lower than expected. The low rate of theses publication is actually seen as an important scientific problem in many developing countries, and even in developed countries [2]. These rates vary from country to country and have been reported as 17% in France, 17.6% in Peru, 23.8% in Finland, and 30% in India [3]. In Turkey, these rates are much lower [4, 6, 7]. The publication rate for theses in the Anaesthesiology and Reanimation speciality in Turkey was 11.3% [5]. However, this study only considered 1207 theses produced from universities. In our study, although the publication rate of theses was relatively high (39.1%), it is still somewhat disappointing. We believe that the obligation to publish the theses introduced by the Inter-University Board (IUB) in 2018 has greatly contributed to this increase.

In addition to compelling reasons such as excessive workload, lack of sufficient education,

the requirement to publish in foreign languages, and the low acceptance rate of journals with a high impact factor, the requirement for publication in SCI or SCI-E indexed journals in the Turkish Associate Professorship criteria also caused both a decrease in the rate of publication and a prolonged delay in publication. This delay in publication has previously been reported to be 3.59±2.96 years, 2.9±2.31 years and 3.3 years [5, 8, 9]. In our study, in keeping with previous reports, the mean delay to journal publication was 3.46±2.62 years.

Anesthesiology and Reanimation is a multidisciplinary field of expertise that includes many areas. Hence, there are many subjects to be researched. There are broad research areas, such as general anesthesia, regional anesthesia, peripheral nerve blocks, algology, and intensive care. According to our findings, general anesthesia was the most studied area, and general anesthesia was also the most frequently published thesis topic. These results are similar to those of Yılmaz et al [5].

In accordance with the Associate Professorship criteria used in our country, the obligation to publish in SCI or SCI-E listed and other internationally indexed journals means that authors look first to these journals rather than national journals. This may explain the high publication rates in SCI/SCI-E and other international journals, both in the literature and in our article. (36.7% SCI/SCI-E and 36.3% other international index vs 23.3% Ulakbim/TR index).

In the literature, particularly in the field of Anesthesiology and Reanimation, the number of female authors has increased over the years. Pagel et al. reported the rate of female authors to be 22.9% [10], while it was 31.3% in a second study from the same group [11], both published in 2019. We found the rate of female authorship to be much higher at 51.9%, possibly reflecting gender differences in medical recruitment between Turkey and the USA.

The difference in the publication rate (6.6% vs 1.3%) between the university and the state hospital in the study conducted by Özgen et al. in 2011 [4] was not seen in our study. We found that the rate of publication of university- and Ministry of Health-based theses increased, and there is no difference between them (39.5% vs 36.5%). In addition, the publication rate of theses of those who went on to an academic career was found to be significantly higher.

Finally, our study showed that in the group of those who published their thesis, those who received specialization from the Ministry of Health were more likely to go on to an academic career than those who received specialization from a university (p=0.002).

Our study has some limitations. First, only theses on the CHE Thesis database and to which we were granted access were included. Second, theses that were only published in scientific congresses, were not accepted in the publication category. Third, as an academic career, Dr. Faculty member, Associate Professor, and Professor was included, minor major acquirers are not evaluated in the academic section. However, who has the titles such as Dr., Research Assoc., Assoc. Prof., in his/her minor majors, are included in the study.

This study showed that many theses are never published in a scientific journal and the valuable scientific data they contained therefore remains widely inaccessible. The scientific quality of theses requires improvement, and scientific institutions should take adequate steps to increase their scientific value. This should result in higher international journal publication rates and improve access of all to data obtained during scientific thesis research. We hope that these barriers will be overcome by allowing medical assistants to devote more time to develop their academic skills, perhaps by adding educational programs to write a scientific paper into the basic medical syllabus.

CONCLUSION

Although higher than previously reported, the publication rate of theses was still less than half and the average delay before journal publication was close to 3.5 years. Encouragingly, the proportion of female authors had increased, theses written under the auspices of university or the Ministry of Health were published at the same rate, most theses were published in international indexed journals, and the thesis owner was usually the first author in the journal article.

Author contribution

Study conception and design: İK, MYK, and AK; data collection: İK, MYK, and AK; analysis and interpretation of results: İK, MYK, and AK; draft manuscript preparation: İK. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

It was decided by the Clinical Research Ethics Committee of Kocaeli Derince Education and Research Hospital that the approval of the ethics committee was not required, since the theses allowed by the authors were examined.

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Conflict of interest

The authors declare that there is no conflict of interest.

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acta medica

ORIGINAL ARTICLE

Evaluation of Patients with Diarrhea Applying to the Outpatient Gastroenterology Clinic of Research Hospital

Çağlayan Merve Ayaz[1]
ORCID: 0000-0003-2574-8683

Batuhan Başpınar[2]
ORCID: 0000-0003-3143-2642

Ertuğrul Kayaçetin[2]
ORCID: 0000-0002-8822-3991

[1]Department of Infectious Diseases and Clinical Microbiology, Ankara City Hospital, Ankara, Turkey.

[2]Department of Gastroenterology, Ankara City Hospital, Ankara, Turkey.

Corresponding Author: Çağlayan Merve Ayaz
Infectious Diseases and Clinical Microbiology Clinic, Ankara City Hospital, Ankara, Turkey.
E-mail: merve.ayz@hotmail.com

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ABSTRACT

Objectives: Diarrhea is a common health problem and may occur for many infectious and non-infectious causes. In this study, it was aimed to investigate the causes, methods used in diagnosis and the results obtained in patients who applied to the gastroenterology clinic with the complaint of diarrhea.

Materials and methods: 187 patients who presented with diarrhea between 01.11.2019-01.11.2020 were included in this study.

Results: Acute diarrhea was detected in 32 (17.1%) out of 187 patients, persistent in 34 (18.2%), and chronic diarrhea in 121 (64.7%). The cause of diarrhea was detected in 148 (79.1%) patients. Infectious cause in 66 (%44.6) patients; inflammatory bowel disease (IBD) in 41 (27.7%) patients; irritable bowel syndrome (IBS) in 22 (14.9%) patients and less frequently as other diagnose were listed. The cause of diarrhea was detected in 73.6% of patients with chronic complaints, and this rate was 87.5% in acute diarrhea; and 91.2% of those presenting with persistent diarrhea ($p = 0.04$). Lower C reactive protein levels were found in irritable bowel syndrome compared to other diarrheal causes ($p<0.001$). It was observed that anti-infective treatment was used more frequently in acute and persistent diarrhea compared to chronic diarrhea ($p <0.001$).

Conclusion: Although application to outpatient clinics were more frequent due to chronic diarrhea, acute and persistent diarrhea were also not rare (35.3%). The reason to explain diarrhea has been found in the majority of patients. Infectious induced diarrhea was seen as the most common cause, it was followed by IBD and IBS, respectively. When prescribing anti-infective agents, clinical, laboratory and microbiological results should be considered and inappropriate drug use should be avoided.

Keywords: Diarrhea, Gastroenterology, Anti-Infective Agents

INTRODUCTION

Diarrhea is a common problem characterized by soft, watery stools and increased frequency of bowel movements. Etiology can stem from many infectious or non-infectious causes [1]. Although it usually does not last longer than a few days, in patients with prolonged diarrhea irritable bowel syndrome (IBS), chronic infections, systemic diseases (such as hyperthyroidism, diabetes), inflammatory bowel diseases (IBD), malignancies, celiac disease and specific enzyme deficiencies should be investigated [2,3].

Acute infectious diarrhea is the 5th most common cause of death from all causes in the World [4]. It is usually self-limited. Only some infections require anti-infective therapy. Appropriate use of diagnostic tests and treatments minimizes potentially unnecessary costs, reduces adverse events,

optimizes clinical outcomes, and limits antibiotic resistance [5]. Diarrhea lasting longer than 14 days but less than four weeks is classified as persistent diarrhea and bacterial and protozoal infections often take place in the etiology [6,7]. Chronic diarrhea (≥ four weeks) affects approximately 5.0% of the population at a given time and is a common problem often caused by non-infectious causes in developed countries [2].

When the underlying diseases, symptoms, examination findings, and histories of the patients are combined, acute diarrhea can be diagnosed in most of the cases. However, advanced diagnostic methods may need to be used in persistent and chronic diarrhea [2]. Stool culture, examination of fresh stool sample under light microscopy, investigation of viral agents, blood tests (hemogram, kidney and liver function tests) and inflammatory markers such as C reactive protein (CRP), erythrocyte sedimentation rate (ESR) are used for initial work-up. Imaging and endoscopic methods are also used when necessary.

Although treatment constitutes the symptom-relieving medications in most cases, Antibacterial, antiprotozoal and anthelmintic drugs can be used in selected cases. In non-infectious diarrhea, treatment of the underlying causes should be employed. In this study, it was aimed to investigate the epidemiological history, causes of diarrhea, methods used in diagnosis and treatments given to patients who applied to the gastroenterology clinic with diarrhea.

MATERIALS AND METHODS

Study Design

This retrospective cross-sectional study was conducted in gastroenterology clinic Ankara City Hospital. Patients who applied to the Gastroenterology clinic between Nov,1, 2019 and Nov,1, 2020 with the complaint of diarrhea were examined. A total of 599 patients were evaluated for eligibility for the study. Among these patients, 187 patients aged ≥18 years who presented with diarrhea were included in the study (Figure 1). Patient data were obtained from the hospital automation system. The data obtained from the hospital automation system for the patients involved in the study include age, gender, presence of concomitant disease, duration and nature of diarrhea, recent use of new drugs, history of eating out, presence of the same symptom in family members, biochemical, microbiological, serological and pathological studies, imaging methods, presence of an interventional procedure due to diarrhea, the cause of diarrhea determined as a result of the examinations, and the drugs preferred in the treatment are included.

Stool samples evaluated macroscopically in terms of color, consistency, quantity, form, odor, blood and presence of mucous. Microscopic examination is a diagnostic tool for defining protozoa, helminths, and fecal leukocytes, erythrocytes. Fresh stool sample was used for detecting for motile organisms (parasites, helminths, cysts and trophozoites).

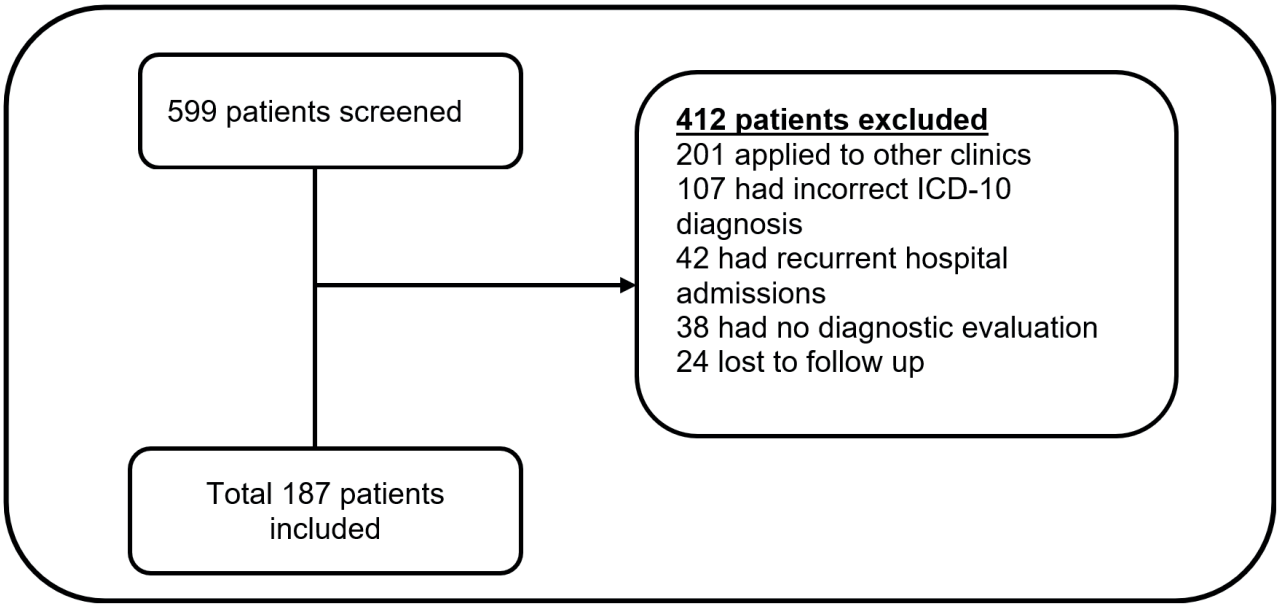

Figure 1. Study profile

A stool culture method was used to identified bacterial and fungal causes. A stool antigen test (monoclonal immunoassays) for detection of *Helicobacter pylori* was performed for making the diagnosis. Nucleic acid tests such as polymerase chain reaction (PCR) were selected to detect *rotavirus* in the stool. Stool samples of only patients with diarrhea should be studied and no checking should not be performed after treatment. Most *Clostridioides difficile* strains produce both A and B toxins, but some strains produce only A toxin or B toxin. Toxin B is clinically important. Monoclonal immunoassays test performed for both toxins [8].

Leukocytosis, defined as an elevated white blood cell (WBC) count greater than 12,000/μL, and WBC count of <4,000/μL was considered leukopenia. C reactive protein >5 mg/L; ESR >20 mm/hr, lactate dehydrogenase >245 U/L, alanine transaminase (ALT) >50 U/L and aspartate transaminase (AST) >35 U/L were considered higher than normal.

Ethical permission of the study was obtained from Ankara City Hospital Clinical Research Ethics Committee (06.01.2021/20-1344).

Statistical Analysis

SPSS Statistics 24 (IBM, New York, ABD) program was used in the comparison of statistical data. Descriptive statistics were expressed as numbers and percentages for categorical variables and as mean, ± standard deviation, median, and minimum-maximum for numerical variables. Pearson chi-square and Fisher's Exact test for comparison of categorical data between groups; Kruskal-Wallis tests were used for comparisons between independent groups for numerical variables that were not normally distributed, respectively. The statistical significance level in the analyzes was accepted as p value < 0.05.

RESULTS

Given the eligibility for the study, 599 patients were screened and 187 patients who met the inclusion criteria were included in the study. Of 187 patients, 104 (55.6%) were male and 83 (44.4%) were female. The median age was 38.0 (min:18.0-max: 81.0).

Underlying disease was present in 127 (67.9%) patients. Diabetes (6.4%) and hypertension (5.4%) were most common. Nine (4.8%) patients had a history of new drug use for the treatment of underlying diseases. Immunosuppression was present in 8 (4.3%) patients and these patients were using immunosuppressive drugs due to underlying malignancy, rheumatological disease or organ transplantation.

All of the patients (100.0%) were questioned for presence of similar symptoms in their family, suspicious food and water consumption, but no similar complaints were detected in the family or in the environment of any patient. It was found that only 1 (0.5%) patient had a history of eating out.

When the patients are evaluated according to the duration of diarrhea; acute diarrhea was detected in 32 (17.1%) patients, persistent in 34 (18.2%) patients, and chronic in 121 (64.7%) patients.

In the macroscopic examination of stool, 128 (68.4%) stool of patients were bloodless-mucous-free; bloody-mucous diarrhea was observed in 28 (15.0%) patients. In microscopic examination, leukocytes and erythrocytes were detected in 59 (31.6%) and 24 (12.8%) patients, respectively. Of 162 (86.6%) patients for whom fresh stool samples were requested, protozoal parasites were seen in 14 (8.6%) out of them (8 *Blastocystis spp* cysts, 3 *Entamoeba histolytica* cysts, 3 *Giardia intestinalis* cysts) and increased yeast was seen in 3 (1.8%) out of them. There was growth in only one patient's stool sample sent for culture (*Salmonella spp*).

Helicobacter pylori antigen test and *Clostridioides difficile* toxin B were positive in 12 (6.4%) and in 9 (4.8%) patients, respectively.

Cytomegalovirus (CMV) (>80 copies/mL) was detected in blood of four patients and *rotavirus* was detected in stool of two patients by PCR. The comparison of the variables according to the duration of diarrhea was presented in Table 1.

Acute kidney injury secondary to diarrhea developed in 4 (2.1%) patients. Abdominal ultrasound and computed tomography (CT) were performed, if needed, and pathological findings related to the intestines were thickening of the intestinal wall, free fluid between the intestines, dilatation and edema in the intestinal loops. No diagnostic result could be reached in the abdominal CT of 8 (27.6%) patients.

Table 1. Characteristics of patients by duration of diarrhea[α]

	Acute diarrhea (n, %)	Persistent diarrhea (n, %)	Chronic diarrhea (n, %)	p value
Age, years (min-max) (n=187)	36.0 (18.0-77.0)	44.5 (21.0-81.0)	37.0 (18.0-81.0)	0.38
Sex (female) (n=187)	15 (46.9)	13 (38.2)	55 (45.5)	0.72
Underlying disease* (n=187)	16 (50.0)	10 (29.4)	33 (27.3)	0.046
Immunosupression* (n=187)	1 (3.1)	4 (11.8)	3 (2.5)	0.07
New drug use* (n=187)	3 (9.7)	-	6 (5.0)	0.15
Fresh wet stool examination* (n=167)				
Protozoa*	2 (14.3)	5 (35.7)	7 (50.0)	0.21
Erythrocyte*	4 (15.4)	4 (12.5)	16 (14.7)	0.94
Leukocyte*	12 (46.2)	13 (40.6)	34 (31.2)	0.28
Clostridioides difficile toxin B* (n=88)	1 (7.1)	3 (25.0)	5 (8.1)	0.16
Leukocytosis WBC>12,000/μL* (n=183)	5 (16.7)	4 (11.8)	20 (16.8)	0.77
Leukopenia WBC<4,000/μL* (n=183)	1 (3.3)	1 (2.9)	3 (2.5)	1.00
ALT>50 U/L or AST>35 U/L* (n=183)	3 (10.0)	2 (5.9)	5 (4.2)	0.40
CRP >5 mg/L* (n=154)	11 (50.0)	15 (53.6)	44 (42.3)	0.51
ESR >20 mm/hr* (n=72)	4 (40.0)	5 (35.7)	10 (20.8)	0.31
LDH >245 U/L* (n=142)	5 (23.8)	5 (17.9)	11 (11.8)	0.33
Helicobacter pylori* (n=48)	2 (28.6)	1 (20.0)	9 (25.0)	1.00
Abdominal ultrasound[β] (n=78)	3 (33.3)	1 (8.3)	8 (14.0)	0.31
Abdominal CT[β] (n=29)	-	3 (60.0)	18 (75.0)	NA
Abdominal MRI[β] (n=17)	1 (50.0)	-	11 (73.3)	NA
Endoscopy[β] (n=65)	3 (75.0)	6 (85.7)	40 (74.1)	0.85
Colonoscopy[β] (n=104)	9 (90.0)	12 (80.0)	51 (64.6)	0.17
Biopsy[β] (n=84)	7 (100.0)	9 (81.8)	59 (89.4)	0.66
Cause of diarrhea[x] (n=187)	28 (87.5)	31 (91.2)	89 (73.6)	**0.04**
Use of antimicrobial drug* (n=166)	20 (62.5)	20 (62.5)	32 (31.4)	**<0.001**

[α]Numbers and percentages belong to columns. *Represents the existence of the specified variables. [β]Represents the presence of pathological result. [x]Represents patients whose cause can be found.

n: number, %: percent, min: minimum, max: maximum, WBC: White blood cell, ALT: Alanine transaminase, ASR: Aspartate transaminase, CRP: C reactive protein, ESR: Erythrocyte sedimentation rate, LDH: Lactate dehydrogenase, CT: Computed tomography, MRI: Magnetic resonance imaging, NA: Not applicable.

Of the patients who underwent endoscopy, 20 (30.8%) had antral gastritis, 19 (29.2%) had pangastritis and 16 (24.6%) had normal findings. Duedonitis, alkaline reflux gastritis and esophagitis were detected less frequently or accompanied by other diagnoses. Of the patients who underwent colonoscopy, 32 (30.7%) had normal findings, 21 (20.2%) had ulcerative colitis, 11 (10.6%) non-specific colitis, 10 (9.6%) adenoma/polyp, 8 (7.7%) had ileocolitis. Diverticular disease, edematous appearance, hemorrhoids and mass were detected less frequently or accompanying other diagnoses.

Biopsy was performed in 84 (44.9%) patients who underwent endoscopy and colonoscopy, if clinically necessary. Of the biopsy results of the patients, 15 (17.9%) had ulcerative colitis, 11 (13.1%) had colitis that cannot be classified as pathologically, 11 (13.1%) had *H. pylori*, 11 (13.1%) had chronic gastritis, 9 (10.7%) had Crohn's disease and 9 (10.7%) had normal findings. Ileitis, collagenous colitis, adenoma/polyp, duodenitis, adenocarcinoma, findings compatible with celiac disease, and edema in the colon were detected less common or accompanying other diagnoses.

The cause of diarrhea was detected in 148 (79.1%) patients as a result of clinical findings, and laboratory, microbiological, pathological examinations, and the cause could not be found

Table 2. The causes of diarrhea (n=148)

	n (%)		n (%)
Infectious causes*	66 (44.6)	**Irritable bowel syndrome**	22 (14.9)
Protozoa	14 (21.2)	**Others***	
Clostridioides difficile	9 (13.6)	Celiac disease	5 (3.4)
Rotavirus	2 (3.0)	Malignancies	4 (2.7)
Salmonella spp	1 (1.5)	Drug-related diarrhea	3 (2.0)
Thought to be infectious	42 (63.6)	Adenoma/polyp	3 (2.0)
Inflammatory bowel diseases*	41 (27.7)	Chronic pancreatic insufficiency	2 (1.4)
Ulcerative colitis	23 (56.1)	Collagenous colitis	2 (1.4)
Crohn's disease	13 (31.7)	Indeterminate colitis	1 (0.7)
Indeterminate IBD	6 (14.6)	Anatomical dysfunction	1 (0.7)
		Diarrhea after coronavirus	1 (0.7)

*Some of the patients have more than one cause.

n: number, %: percent, IBD: Inflammatory bowel diseases.

Supplementary Table 1. Change of laboratory parameters according to diarrhea causes[a]

	Infectious causes n (%)	Inflammatory bowel diseases n (%)	Irritable bowel syndrome n (%)	Others * n (%)	p value
Leukocytosis (n=143)	11 (17.7)	10 (25.0)	1 (4.8)	1 (5.0)	0.11
Leukopenia (n=143)	2 (3.2)	1 (2.5)	-	2 (10.0)	0.41
Elevated CRP (n=122)	24 (44.4)	26 (70.3)	1 (6.3)	8 (53.3)	<0.001
Elevated ESR (n=58)	6 (33.3)	10 (41.7)	-	2 (25.0)	0.17
Elevated transaminase levels (n=143)	5 (8.1)	1 (2.5)	1 (4.8)	1 (5.0)	0.78
Elevated LDH (n=110)	9 (18.8)	4 (12.9)	2 (12.5)	2 (13.3)	0.90

[a]Numbers and percentages belong to columns. *Others: Celiac disease, malignancy, drug-related diarrhea, adenoma/polyp, chronic pancreatic insufficiency, collagenous colitis, indeterminate colitis, anatomical dysfunction, and prolonged diarrhea after coronavirus.

n: number, %: percent, CRP: CRP: C reactive protein, ESR: Erythrocyte sedimentation rate, LDH: Lactate dehydrogenase.

in the remaining 39 (20.9%) patients. The cause of diarrhea was considered to be infectious in 66 (44.6%) patients [demonstrated by diagnostic methods in 22 (14.9%) patients - CMV positive patients were not included in this group because they did not have colonoscopic findings]; IBD in 41 (27.7%) patients and IBS in 22 (14.9%) patients. Celiac disease, malignancy, drug-related diarrhea, adenoma/polyp, chronic pancreatic insufficiency, collagenous colitis, indeterminate colitis, anatomical dysfunction, and prolonged diarrhea after coronavirus were detected less frequently or accompanying other diagnoses. The causes of diarrhea was presented in Table 2.

The laboratory results of the patients were evaluated according to the causes of diarrhea, and no difference was found in leukocytosis, leukopenia, ESR, transaminase and LDH values. There was only difference in CRP values ($p<0.001$). When subgroup analyzes were made, it was seen that this difference was due to the low CRP values in IBS patients. The variation of laboratory parameters according to the causes of diarrhea was presented in Supplementary Table 1.

Treatment was given to 113 (60.0%) of the patients presenting with diarrhea, and anti-infectives were included in the prescriptions written to 72 (63.7%) patients. Anti-infective preferences were given to the patients were ciprofloxacin and metronidazole in 30 (41.66%) patients; only metronidazole in 25 (34.7%) patients; only ciprofloxacin in 5 (6.9%) patients; amoxicillin-clavulanic acid and clarithromycin in 3 (4.2%) patients; tetracycline and metronidazole in 3 (4.2%) patients and rifaximin in 2 (2.8%) patients, respectively. There were 1 (1.4%) patient each given albendazole, ciprofloxacin and ornidazole, rifaximin and ornidazole, and nifuroxazide. Except for anti-infective drug, mesalazine in 35 (30.9%) patients; steroid in 14 (12.4%) patients; azathioprine was preferred in 9 (7.9%) patients, respectively. Symptomatic treatments were used in the remaining patients.

DISCUSSION

Acute and persistent diarrhea occur more frequently due to infectious causes and are more common in low- and middle-income countries where sanitation is inadequate [1]. Chronic diarrhea, on the other hand, is distinguished from others by its duration. Patients with chronic diarrhea usually require additional investigations, but in some, the history and physical examination may be sufficient to guide treatment. For example, diet, medications, surgery, radiation therapy, and IBS can be distinguished from the patients' history. Testing may be required when alarm symptoms are present (eg, weight loss, bloody stool), when there is no obvious cause, or for differential diagnosis [2]. In our study, patients who applied to the gastroenterology outpatient clinic with diarrhea were evaluated. The applications with chronic diarrhea were higher as expected (121 patients, 64.7%). Considering that patients with acute or persistent diarrhea often apply to infectious diseases, family medicine and internal medicine clinics or emergency department, the rate of acute or persistent diarrhea in 35.3% of the patients presenting to the gastroenterology clinic can be considered high.

A concomitant disease was observed in 16 (50%) patients who presented with acute diarrhea. While self-limiting acute diarrhea in adults usually does not require hospital admissions [1,9], the high rate in our study can be explained by the fact that patients behave cautiously and apply to hospitals more frequently in the presence of concomitant disease.

The cause of diarrhea was detected in 73.6% of the patients with chronic diarrhea, 87.5% of the patients with acute diarrhea, and 91.2% of the patients with persistent diarrhea. The difference may be due to not evaluating all causes and investigate accordingly in chronic diarrhea in which less common causes can be seen [3]. Bile acid and carbohydrate malabsorption, chronic idiopathic secretory diarrhea, fecal incontinence, functional and iatrogenic diarrhea, autonomic neuropathy, peptide-secreting tumors, immunodeficiencies, microscopic colitis, amyloidosis, dermatological and endocrinological diseases should be considered in selected patients and investigation should be performed accordingly [2].

In 39 (20.9%) of the patients, the cause of the diarrhea could not be determined. Insufficient history taking (eg, intolerance to specific nutrients, diet, alcohol use, history of surgery, presence of radiotherapy, family history etc.), physical examination, inadequate use of diagnostic methods (eg, electrolyte search in stool, evaluation of stool composition, lack of pathological examination of the colon mucosa etc.) or patients' non-compliance with the requested examinations (eg, refusal of colonoscopic evaluation etc.) were observed as the causes of undiagnosed cases.

Although an infectious cause (bacteria, protozoa and/or virus) was detected in 12.8% of 108 patients, anti-infective drugs were used in 38.5% of the patients. The rate of anti-infective use in acute and persistent diarrhea is 62.5%. Although an infectious causes were shown only in 8.3% of 121 patients with chronic diarrhea, anti-infective treatments were used in 31.4% of the patients. This situation can be expected considering acute and persistent diarrhea in Turkey. However, it can be clearly observed that anti-infective drug use is significantly high in patients with chronic diarrhea. Mostly prescribed anti-infective agents for chronic diarrhea were metronidazole in 75.0% (24 out of 32 prescriptions) and ciprofloxacin in 37.5% (12 out of 32 prescriptions). Although the high rate of metronidazole use is thought to be stemmed from possibility of a chronic infection, these high rates of ciprofloxacin treatment could not be attributed to a rational reason. In two different guidelines, which compiled the causes of chronic diarrhea, it was stated that chronic diarrhea of infectious origin is a rare condition in developed countries, but anti-infective use is appropriate in cases where the agent is indicated [10,11]. Among the reasons for high rates of anti-infective use, physicians' tendency to prescribe anti-infective agents even in the absence of an evidence of infection in patients with acute or persistent diarrhea [1,7]. This situation can be attributed to the fact that Turkey is a developing country and physicians may want to rule out infectious causes in the differential diagnosis process by prescribing anti-infective agents. However, this situation not only causes inappropriate anti-infective use, but also leads to drug-related side effects and increased drug resistance.

In patients who cannot be diagnosed with anamnesis and physical examination, it was observed that requesting full blood count, LDH, ESR, and transaminases did not make an additional contribution to the diagnosis. Only the CRP value might be helpful in the differential diagnosis of IBS in which CRP values were within normal levels in 93.7% of the patients [12,13]. While laboratory testing is not needed in most of the patients presenting with acute diarrhea, the type of the laboratory test should be selected in accordance with the patient history and clinical presentation in persistent and chronic diarrhea, and the habit of ordering unnecessary laboratory tests for each patient should be avoided as much as possible.

There were some limitations in the presented study. Firstly, there were data deficiencies due to the retrospective nature of the study. Secondly, the study employed a small number of patients, and therefore generalizability of its results to national level could not possible. Lastly, viral agents other than rotavirus were not investigated.

Chronic diarrhea is among the frequent reasons for admission to gastroenterology clinics, but acute or persistent diarrhea is also frequently among the reasons for outpatient referral. Although the cause of diarrhea was found in most of the patients presenting with diarrhea, the cause could not be determined in some patients at the time of admission. Prescribing anti-infectives should be avoided as much as possible in patients whose infectious cause cannot be determined. All differential diagnoses should be screened patiently with anamnesis, physical examination, imaging, appropriate stool and blood tests. When such an approach is taken, the number of patients whose cause of diarrhea cannot be found would gradually decrease.

Although diarrhea is a common health problem that affects many regions around the world, studies comparing acute, persistent and chronic diarrhea and evaluating patients' demographic, clinical, laboratory, imaging and biopsy results, and treatments and responses are relatively few. There is a need for detailed and extensive research in this area.

Author contribution

Study conception and design: ÇMA, BB, and EK; data collection: ÇMA and BB; analysis and interpretation of results: ÇMA and BB; draft manuscript preparation: ÇMA, BB, and EK. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Ankara City Hospital Clinical Research Ethics Committee (Protocol no. 20-1344/06.01.2021).

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Conflict of interest

The authors declare that there is no conflict of interest.

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acta medica

ORIGINAL ARTICLE

Enzymatic Digestion of Fresh-Frozen Human Cornea After Riboflavin/Ultraviolet-A Collagen Crosslinking

Ayşegül Fırat[1]
ORCID: 0000-0001-5105-0057

Züleyha Yalnız Akkaya[2]
ORCID: 0000-0003-3817-2214

Özlem Dikmetaş[3]
ORCID: 0000-0001-5670-2384

Hatice Mürvet Hayran[4]
ORCID: 0000-0001-6058-6304

[1]Department of Anatomy, Hacettepe University Faculty of Medicine, Ankara, Turkey.

[2]Department of Ophthalmology, University of Health Sciences, Ankara Training and Research Hospital, Ankara, Turkey.

[3]Department of Ophthalmology, Hacettepe University Faculty of Medicine, Ankara, Turkey.

[4]Department of Anatomy, Economy University Faculty of Medicine, Izmir, Turkey.

Corresponding Author: Ayşegül Fırat
Department of Anatomy, Hacettepe University Faculty of Medicine, Ankara, Turkey.
E-mail: aysfirat@hacettepe.edu.tr

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ABSTRACT

Objective: Crosslinking (CXL) of the cornea by using riboflavin (RBF)/ ultraviolet-A (UVA) has been developed for stiffening the collagenous matrix of the cornea. Collagenases and other metalloproteinases are known to play an important role in the pathogenesis of corneal ulceration. Our aim in this study was to show and compare the morphological and histopathological changes of the CXL human corneas against enzymatic digestion by collagenase, trypsin and pepsin solutions.

Materials and Methods: Eighteen corneas from nine fresh frozen cadavers were included in this study. The crosslinked group (n=12) was treated with the combined riboflavin (RBF) and ultraviolet-A (UVA) and the control group (n=6) was only treated with RBF. After treatment corneas were exposed to collagenase, trypsin and pepsin enzymes. For each enzyme group one cornea was evaluated morphologically and the other one was evaluated microscopically.

Results: The collagenase treated control group, digestion of the cornea was noted in the first days and completed on the 5th day. The control cornea treated with pepsin was fully digested after 14 days. In the trypsin treated CXL cornea complete digestion was noted after 18 days. In both groups, enzyme activity was observed to be parallel to each other morphologically, but CXL cornea was evaluated to be more resistant to enzymatic digestion.

Conclusion: In conclusion, CXL increases resistance of the cornea against enzymatic digestion. In addition to the biomechanical support, this study was also showing the histopathological changes of CXL procedure and the results of enzymatic digestion, supporting new treatment options in the corneal diseases.

Keywords: Corneal crosslinking, collagenase, pepsin, trypsin

INTRODUCTION

Corneal crosslinking (CXL) causes an increase in the biomechanical strength of cornea by increasing collagen fibril diameter and stiffening the collagenous matrix, leading to an additional band of intense polymer in electrophoresis [1]. Enzymatic digestion is an indicator of biomechanical support of CXL cornea. Relevantly, CXL porcine corneas have been demonstrated to be resistant to collagenase, trypsin and pepsin digestion [2-4]. Since collagenases play an important role in corneal ulceration in different conditions such as infective keratitis, chemical burns or peripheral ulcerative keratitis; increasing the resistance of the cornea against the effect of these enzymes can be achieved by CXL [5-7]. Thus, in this study we aimed to evaluate the resistance of CXL human cadaveric cornea against enzymatic digestion.

MATERIALS AND METHODS

The study was carried out in the Department of Anatomy, Hacettepe University Faculty of Medicine. In this study 18 corneas of nine fresh-frozen human cadavers were used. Six of the corneas were reserved for the control group and 12 of them were included in the CXL group. Before the corneas were trepanized, a deep-lysed riboflavin (RBF) solution was applied to the cornea (3-4 drops every 2 minutes) for 20 minutes in the control group. In the CXL group same procedure (3-4 drops of Rbf every 2 minutes) was followed by UVA irradiation (CBM Vega X-Link CSO srl, Scandicci, Firenze, Italy) 1 cm away from the cornea at 370 nm wavelength (3 mw / cm2) for 30 minutes. During irradiation, RBF was dropped every 5 minutes to increase the sensitivity of the cornea to the beam. The corneas were then trepanized with an 8.25 mm trepan and immediately placed into seperate glass containers with collagenase, trypsin and pepsin enzyme solutions. One sample of each group was selected for morphometric evaluation and others were prepared for light microscopic examination. Among six corneas included in the control group, selected two were treated for each enzyme: one cornea was photographed for morphometric changes (diameter, slope, digestion and transperancy) in the first, third and fifth days of the experiment; the other one was divided into three equal parts for enzymatic treatment. For each of the enzyme treated corneas in the CXL group; one cornea was photographed for morphometric parameters and others were evaluated microscopically in the first, third and fifth days. Collagenase-A (0.1 mg / ml colA) solution was made up of 7.9 mg of collagenase (Roche, Mannheim, Germany, EC 3.4.24.3) in 20 ml of phosphate buffer solution (PBS). Working pepsin solution (0.4%) was prepared by 80 mg of purified pepsin (Sigma, Munich, Germany, EC 3.4.23.1) in 20 ml of 10 mM HCl at optimum pH=1.5 for pepsin activity. Trypsin working solution with a 0.125% trypsin (Thermo scientific, Cheshire, UK, TA-015-TR) was made up of 1:3 trypsin in PBS buffer (5 mlt 0.125% trypsin+15 mlt PBS). Before incubation with trypsin, protein heat denaturation was performed by boiling the cornea in distilled water at 100 degrees for 10 minutes [2]. Then the corneas were taken into glass tubes and the enzymes were added immediately at room temperature. Slope and diameter measurements were made by using a millimetric paper. In order to measure the slope, dome of the cornea was placed on the slide and the height of the corneal edge from the slide was measured (Fig 1). A four grade scoring scale was performed for transparency and digestion (Four points given for the most transparent and intact corneas, zero point for the least transparent and digested corneas).

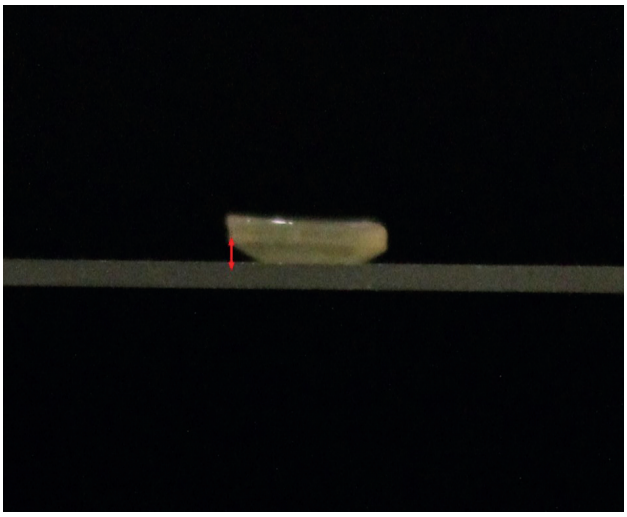

Figure 1. Slope of the CXL cornea after two days of digestion.

For histological evaluation, samples in different enzyme groups were fixed in 2.5% gluteraldehyde (Agar Scientific Ltd., Essex, UK, R1010) for 24 h, at room temperature. Then, samples were post-fixed in 1% osmium tetroxide (Millipore Sigma, Burlington, MA, USA, 56H1140) at 4 °C for 2 h. Following post-fixation tissue samples were dehydrated and then embedded in epoxy resin (Araldite CY212 kit, Agar Scientific Ltd., Essex, UK, AGR 1030). After plastic embedding, approximately 4 µm thick semi-thin sections were cut with a glass knife on an ultramicrotome (LKB Nova Ultramicrotome, Bromma, Sweden). Semi-thin sections were stained with 1% methylene blue (BDH Ltd., Poole, UK, CAS 61-73-4) solution (methylene blue 1 gr + sodium borate 1 g + distilled water 100 ml). Stained sections were examined and captured under the camera Lucida of a Nikon Optiphot (Nikon Corporation) light microscope for histologic analysis (Fig 2).

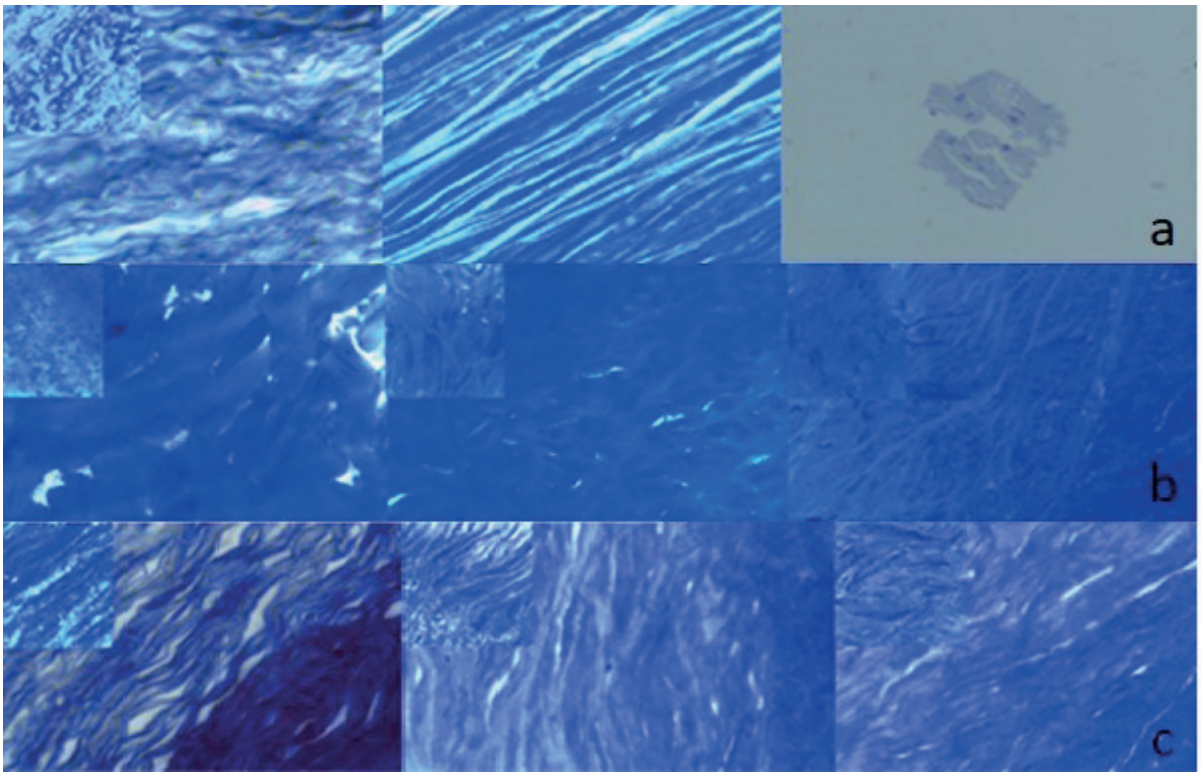

Figure 2. Microscopic evaluation (1st, 3rd and 5th days respectively), a. Corneas treated with collagenase and its control groups on the inset, b. Corneas treated with trypsin and its control groups on the inset, c. Corneas treated with pepsin and its control groups on the inset. Methylene blue staining, x40.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards and approved by the local Ethics Committee (GO 13/275-34).

Statistical analysis

In our study, measurements were made for a total of six observations and two observations (control-CXL) were performed with the same enzyme by a blind observer (AF). We were not able to perform hypothesis tests for comparing enzyme and treatment effects because the sample size was very small. Therefore, statistical evaluation was performed using only the descriptive statistics, and tables and graphs were used instead of hypothesis testing. During follow-up, means were used for morphometric descriptions. In particular, line graphs of first, third and fifth days were considered important for morphologic changes over time and for comparisons with histologic findings (Fig 3,4).

RESULTS

In the collagenase treated group, digestion of the control cornea started immediately and the sample was fully digested on the fifth day. The collagenase treated CXL cornea was also digested rapidly and digestion was completed on the sixth day. The CXL cornea was slightly opaque during the first days of the follow-up, then it became transparent following digestion. Its slope was zeroed in parallel with digestion and its diameter was increased during a process like melting. In both groups, the effects were parallel, but CXL cornea was observed to be more resistant to digestion. Both corneas completed the digestion process not with disintegration but with melting, also without a morphometrical changes were apperent in the transparency (Fig 5).

The control cornea treated with pepsin was fully digested in the 14th day. During the follow-up, the transparency was decreased by half, the slope and the diameter was decreased during the first seven days. After seventh day, the diameter was increased

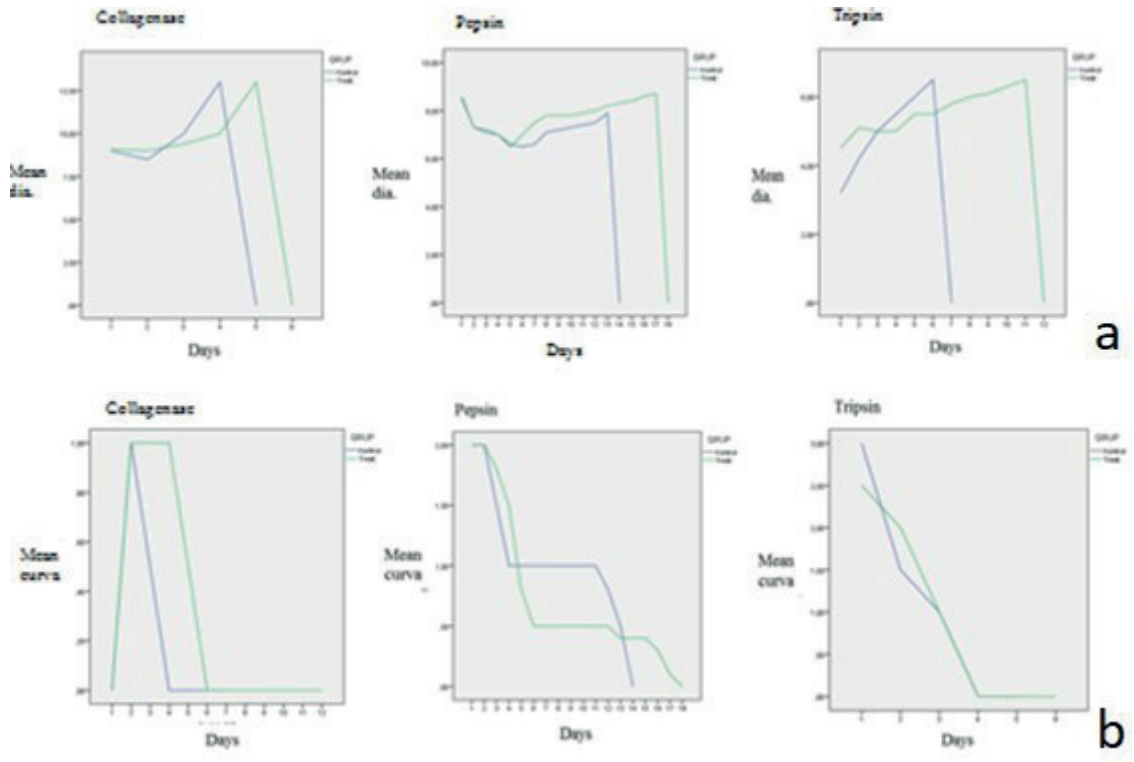

Figure 3. Graphics showing daily changes of a. diameter and b. curvature in control and CXL groups.

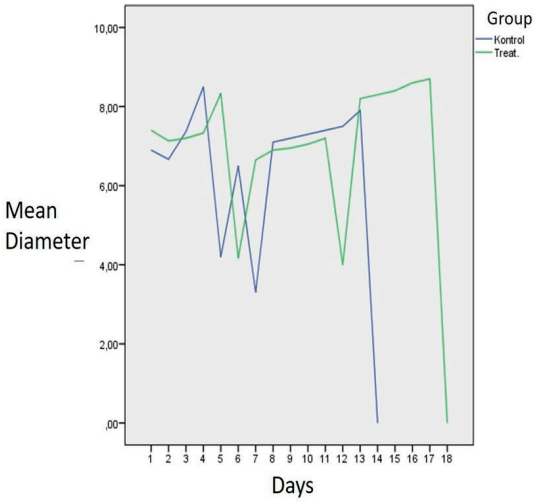

Figure 4. Graphic of the mean diameter change of corneas over time.

because of digestion with melting. The CXL cornea, treated with pepsin was completely digested after 18 days. The diameter of CXL cornea was decreased until the 6th day, then began to increase. Slope of the CXL cornea was decreased throughout the digestion process. In both groups, enzyme activity was observed to be parallel to each other, but CXL cornea was resistant to digestion same as in the collagen treated group. Both corneas were shattered with reduced transparency and completed enzymatic digestion (Fig 6).

Figure 5. Collagenase treated control group (2nd day).

Figure 6. Pepsin treated control group (left) and CXL group (right) (2nd day).

Since the corneas treated with trypsin enzyme were first denaturated by heat, their diameters were reduced by half and their transparency was slightly transparent at the onset of the procedure. The control cornea was disintegrated after six days and complete digestion was noted on the seventh day. In the follow-up of the control cornea, it was observed that its transparency and diameter increased as it approached the day of digestion, and the slope was increased at the beginning and then decreased after the second day of digestion procedure. The CXL cornea treated with trypsin was disintegrated on the eighth day and complete digestion was observed on the 12th day. During the follow-up, it was observed that the initially increased opacity decreased with the digestion process. The slope was increased during the first four days and then decreased and the diameter was decreased regularly throughout digestion. Both corneas were disintegrated into pieces without full transparency and the digestive effects were completed in parallel.

Light microscopic images were also evaluated for the first, third and fifth day and these evaluations were supported by the morphometric results.

The histological evaluation of the control group treated with collagenase was performed only for the first day as digestion and melting was developed very rapidly. On the first day, collagenase impaired the lamellar appearance of the cornea in the control group and the gaps between the fibers were remarkable. On the same day, the lamellar appearance was preserved in the CXL cornea but gaps were observed in some areas. On the third day gaps were increased in number without a disruption on the lamellar organization. A very soft and gelatinous sample was prepared for microscopic evaluation after five days and collagen fibers were observed in these sections. On the first day, the convoluted appearance of the fibers in the cornea was also consistent with the slight decrease in the diameter. During the follow up as cornea became more gelatinous, the diameter and convolutions were both increased.

No gradual increase was observed in the lamellar appearance of collagen in the control and CXL corneas treated with trypsin. The morphology was consistent with the findings in diameter and slope graphs. Until the end of fifth day, uniform lamellar appearance and lack of gaps between fibers were remained almost similar for both groups.

Since the digestive effect of pepsin was noted as the latest, there was no gradual difference in lamellar appearance in the control and CXL corneas for the first days of digestion.

DISCUSSION

Corneal melting occurs in various infectious and non-infectious inflammatory conditions that threaten the vision and the integrity of the eye. It has been shown that the corneal melting is caused by various digestive enzymes [5,7-9]. Thus, in order to save the eye and the vision, the corneal melting must be prevented. This melting processes can be halted by two mechanisms: by decreasing the production of degrading enzymes or by increasing the strength of the cornea to the enzymatic digestion. The later effect is achieved by combined Riboflavin/UVA CXL of the corneal stroma [10-13]. Thus, the corneal stroma becomes more resistant to digestive enzymes from microbial and inflammatory origin [14]. The structural and biomechanical effects of Riboflavin/UVA CXL in the corneal stroma have been evaluated both clinically and experimentally [10-13,15,16]. For in vitro evaluations light, electron and confocal microscopy, x-ray scattering and second harmonic generation imaging, 2-dimensional fast Fourier-transform analysis stress-strain biomaterial tester and enzymatic digestion have been used [17]. The stiffening effect of Riboflavin/UVA CXL on the normal human, rabbit and porcine corneas have been measured by stress-strain biomaterial testers [18-20]. For the first time, Wollensak et al. demonstrated increase in corneal rigidity by 328.9% in 5 human cadaveric corneas, while the increase in 20 porcine corneas was by 71.9% [20]. This biomechanical effect was explained by the increase of 12.2% in collagen fiber diameter due to interfibrillary cross links in rabbit anterior cornea [1]. While 22.6% increase was observed in anterior healthy human cornea, no increase was noted in posterior cornea. In bovine corneas the adherence force (measured with extensometer) of laser in-situ keratomileusis (LASIK) flap after CXL has been

shown to increase and gradually decrease in organ culture afterwards. The authors were not been able to explain this transient effect. The strengthening effect has been shown to occur due to intrafibrillar and interfibrillar crosslinking but not interlamellar [21]. After CXL, a strong band of high molecular-weight collagen polymers that was resistant to mercaptoethanol, heat, and pepsin treatment has been detected by electrophoresis in porcine corneas [3]. As a result of these structural changes, crosslinked porcine and rabbit corneas were found to be more resistant to pepsin digestion. Kanellopoulos et al. evaluated the biomechanical and enzymatic digestion resistance differences between LASIK and LASIK+CXL. The latter showed significant increase in underlying corneal stromal rigidity [22]. Alageel et al. analyzed whether verteporfin with a nonthermal laser increases corneal mechanical stiffness and resistance to enzymatic degradation ex vivo. Corneal resistance to enzymatic collagenase degradation.was notably increased with verteporfin [23]. For the first time Wollensak et al. demonstrated that CXL corneas were dissolved by day 13 and 14 following pepsin and collagenase treatment versus six days in control group in porcine corneas. After heat denaturation, digestion by trypsin was observed by day five and day two respectively in CXL and non-CXL corneas [1]. The anterior curvature was maintained in the first seven days in pepsin and collagenase treated group. A study with all non-CXL corneas (8.5 mm in diameter), complete digestion was noted in six days while the average diameter of CXL corneas decreased by only 12% and the anterior curvature remained visible. In this study, corneas were obtained in 29 hours of death [24]. In another study, three CXL keratoconic corneas, three normal human corneas and three non-treated keratoconic corneas were evaluated. Keratoconic features were changed into normal features during CXL [24]. The samples were then subjected to enzymatic digestion by pepsin. Pepsin was used as it is a nonspecific endopeptidase that can break down both, collagen and proteoglycan core proteins. It is, therefore, more appropriate for assessing the effect of CXL than collagenase [24]. Normally, 90% of the cornea is formed by a stromal layer [25]. This regular thick connective tissue is formed by bundles of collagen, arranged in lamellae. In this study we have seen that CXL procedure increases the digestion time when corneas are treated with collagenase, pepsin and trypsin enzymes. This result explains the biomechanical effect of CXL treatment [19,26-28]. The stroma of the cornea forms about 90% of the thickness of the cornea. This transparent regular connective tissue is formed by bundles of collagen. Therefore, collagenase enzyme has more prominent effects on cornea than the other enzymes that we also had experienced in this study. Regular arrangement of the lamellae is another important feature of the histological appearance of stroma. In the collagenase treated corneas we have seen loss of this regularity and also seen spaces between lamellae as the result of digestion. Excluding the time period between the enzymes and control/ CXL corneas, graphics show that the digestion procedure was parallel in all groups. This means that diameter, curvature, transparency and digestion progresses in the same manner in all enzymes and control/CXL groups. The most apparent difference was the time period of digestion between these groups.

Our results should be interpreted in the light of its potential limitations. The major limitation of the current study is the sample size. The CXL group was treated with the RBF/ UVA and the control group was only treated with RBF. Another limitation is that different types of RBF solutions were not applied and compared. The main strength of our study is that the corneas were obtained from fresh-frozen human cadavers.

These findings also encourage the new treatment techniques of the corneal ulcers. In order to make reliable generalizations and statistics further studies with larger number of samples also evaluating ultrastructural changes of human corneas are needed.

Limitations of the study

The sample size in this study was very small to perform hypothesis tests. Findings should be considered as preliminary results and evaluated using clinical significance. More samples are required to assess statistical significance of the results.

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Author contribution

Study conception and design: AF and ZYA; data collection: AF and ZYA; analysis and interpretation of results: AF, ÖD, and ZYA; draft manuscript preparation: AF, ÖD, ZYA, and HMH. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Ethics Committee of Faculty of Medicine, Hacettepe University (GO 13/275-34).

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Conflict of interest

The authors declare that there is no conflict of interest.

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acta medica

ORIGINAL ARTICLE

Can Syndecan-1 Be Used As A Biomarker In Alzheimer's Disease?

Rana Tuna Dogrul[1]
ORCID: 0000-0001-9470-5113

Cagatay Cavusoglu[1]
ORCID: 0000-0003-4725-8095

Gozde Sengul Aycicek[1]
ORCID: 0000-0002-7118-5589

Cemile Ozsurekci[1]
ORCID: 0000-0002-2373-183X

Hatice Caliskan[1]
ORCID: 0000-0001-9750-3840

Hacer Doğan Varan[1]
ORCID: 0000-0002-5238-1162

Zeliha Gunnur Dikmen[2]
ORCID: 0000-0001-7154-090X

Meltem Gülhan Halil[1]
ORCID: 0000-0001-7597-8140

Mustafa Cankurtaran[1]
ORCID: 0000-0002-8213-7515

Burcu Balam Dogu[1]
ORCID: 0000-0002-4430-6146

[1]Hacettepe University, Faculty of Medicine, Department of Internal Medicine, Division of Geriatric Medicine, Ankara, Turkey.

[2]Hacettepe University, Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkey.

Corresponding Author: Rana Tuna Dogrul
Hacettepe University, Faculty of Medicine, Department of Internal Medicine, Division of Geriatric Medicine, Ankara, Turkey.
E-mail: rana_tuna@hotmail.com

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ABSTRACT

Background: Syndecan-1 (SDC-1) is a member of the syndecan family, which includes heparan sulfate proteoglycans. SDC-1 is important for cell-cell and cell-matrix interactions. The aim of this study is to examine the relationship between serum SDC-1 levels and mild cognitive impairment (MCI) and Alzheimer's disease (AD).

Methods: Eighty-two patients aged 65 years and over were included in the study. The Mini-Mental State Examination (MMSE) was used to evaluate the cognitive functions of the patients. Comprehensive geriatric assessment components were administered to the patients. Serum SDC-1 levels were measured with an enzyme-linked immunosorbent assay kit.

Results: When patients were grouped as control, MCI and AD, significant decreases were observed in Katz daily living activity ($p<0.001$), Lawton instrumental daily living activity ($p=0.001$), Mini-nutritional assessment ($p=0.001$), MMSE ($p=0.001$) scores. SDC-1 level was 154.88±22.85 in the control group, 157.95±19.45 in the MCI group, and 159.54±14.04 ng/mL in the AD group, and no significant correlation was observed ($p=0.677$). When correlation analyzes were performed with SDC-1, a negative correlation was found with the Yesavage geriatric depression scale score (Spearman rho: -0.223 $p=0.044$).

Conclusion: No correlation was found between SDC-1 level and AD, and it showed a negative correlation with depression. Clarifying the pathogenetic processes more clearly will guide the development of new treatment strategies.

Keywords: Syndecan-1, Mild cognitive impairment, Alzheimer's disease, Yesavage geriatric depression scale

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia and one of the leading causes of morbidity and mortality in the aging population. AD is neuropathologically characterized by senile plaques formed by the deposition of extracellular amyloid β protein (Aβ) and neurofibrillary tangles formed by the deposition of intracellular hyperphosphorylated tau protein [1]. Although the precise role of Aβ in AD is not fully understood, plaque formation from accumulated Aβ is still thought to be a central event in disease development. It has been shown that the accumulation of Aβ resulting from the imbalance between production and clearance has a profound effect on the pathogenesis of AD. Although neuronal loss caused by inflammation and toxic mechanisms caused by senile plaques and neurofibrillary tangles are known as the main pathogenetic mechanism, it has not been clearly determined how this accumulation occurs in the brain and how it causes neuron loss [2,3].

The interaction of heparan sulfate proteoglycans (HSPGs) with Aβ is well known. HSPGs play a role in various pathogenic features of AD, including its localization with amyloid plaques. Binding of Aβ1–42 to HSPGs is mediated by electrostatic interactions between negatively charged heparan sulfate chains and the cationic heparin-binding motif of Aβ1–42. The binding of Aβ1–42 to heparan sulfate chains induces its multimerization, leading to the formation of toxic fibrillar aggregates [4,5].

The word "syndecan" derives from the Greek word "syndein" meaning "to connect", thus reflecting its biological role. Due to their highly sulfated polyanionic glycosaminoglycan (GAG) chains, SDCs interact with numerous extracellular cationic ligands and transmit signals from the extracellular space to the cellular interior, affecting cellular metabolism, transport, and information transfer. Syndecan-1 (SDC-1) is one of four members of the syndecan family. It is a cell surface protein consisting of three structural domains that bind heparin sulfates and chondroitin sulfates, one of which is extracellular. It is involved in the regulation of cell proliferation, migration, and organization of the cytoskeleton. As key regulators of cell signaling and biological functions, SDCs also have important roles in the pathogenesis of various diseases [6].

There is currently no treatment to stop or reverse the progression of AD. Therefore, further studies are needed to understand the etiopathogenesis of AD and to identify possible new pathological pathways or markers. To our knowledge, there is no study examining serum SDC-1 levels in patients with AD and comparing levels between patients with normal cognitive function and those with MCI. The aim of this study is to evaluate the relationship between serum SDC-1 levels and AD.

MATERIALS AND METHODS

Patient characteristics

A total of 124 patients aged 65 years and older who applied to the geriatrics outpatient clinic, with informed consent, were evaluated, after the exclusion criteria, 82 patients (32 with AD, 30 with MCI and 20 without cognitive impairment) were included in the study. Patients with the following diseases were excluded in the study: 1) Patients with inflammatory diseases, 2) With active infection, 3) With a diagnosis of malignancy, 4) With a diagnosis of heart failure, 5) With vascular dementia, Parkinson's dementia, Lewy body dementia, frontotemporal dementia and other neurodegenerative diseases. Petersen's criteria were used for the diagnosis of MCI [7]. After comprehensive geriatric assessment (CGA) and cognitive evaluation, the diagnosis of dementia was made according to NINCDS-ADRDA [8] and DSM-V [9] criteria. Cognitive dysfunction severity was determined using a modified version of the Reisberg functional assessment staging (FAST) scale [10]. For the differential diagnosis of each patient diagnosed with dementia, magnetic resonance imaging was performed before the diagnosis of AD. Other types of dementia were ruled out.

The patient's age, gender, with whom they lived, educational status, smoking and alcohol use, body mass index (BMI), concomitant diseases, incontinence, history of falling, and the number of drugs used were recorded. The type of study was designed as a cross-sectional study.

Comprehensive Geriatric Assessment and Cognitive Assessment

Within the concept of CGA some standardized tools were applied. The Katz Activity of Daily Living Scale (ADL) [11] and the Lawton-Brody Instrumental Activities of Daily Living (IADL) [12] scales were used to evaluate the functional status of the patients. The Yesavage Geriatric Depression Scale (GDS) short form was administered to the patients to screen for depression [13]. For malnutrition risk assessment, patients were evaluated with the Mini Nutritional Assessment short-form (MNA-SF) [14].

The cognitive status assessment of the patients participating in the study was performed by clinical evaluation and anamnesis from the patient and the caregivers. Screening instruments including Folstein Mini-Mental State Examination (MMSE), and the clock drawing test [15,16]. After the evaluation, and using the mentioned criteria, the patients were grouped as normal, MCI and AD.

Syndecan-1 Measurement

Blood samples were taken from the patients and centrifuged at 4,000 g for 10 minutes. All samples were stored at –80 C until testing. Serum SDC-1 levels were analyzed by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Human Syndecan-1 CD138 ELISA Kit, Bioassay Technology Laboratory, China) according to the manufacturer's instructions.

The plate is pre-coated with human SDC-1 antibody. SDC-1 present in the sample is added and binds to antibodies coated on the cavities. Biotinylated human SDC-1 antibody is then added and binds to SDC-1 in the sample. Streptavidin-HRP is then added and binds to the Biotinylated SDC-1 antibody. After incubation, unbound Streptavidin-HRP is washed off during a wash step. The substrate solution is then added and color develops in proportion to the amount of human SDC-1. The reaction is terminated by the addition of the acidic stop solution and the absorbance is measured at 450 nm.

Ethics

The study protocol was evaluated and approved by the Hacettepe University Ethics Committee (Ethics committee approval number: GO 17/963). Informed consent was obtained from all the patients.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0. Descriptive statistics were examined using visual (histograms and probability graphs) and analytical methods (Kolmogorov-Smirnov/ Shapiro-Wilk tests) for numerical variables to fit the normal distribution. Descriptive statistics were given using the mean and standard deviation (mean±SD) values for the normally distributed variables, and the median and minimum-maximum values for the non-normally distributed variables. Categorical variables were expressed as numbers and percentages (%). Stundent's t-test and Mann-Whitney U test were used when comparing numerical parameters with normal and non-normal distribution between two independent groups. The Kruskal Wallis test was used when comparing numerical variables that did not show normal distribution with more than two independent groups. One-way Anova test was used to compare the normally distributed numerical parameters with more than two independent groups. The homogeneity of variances was determined using Levene's test. Post-hoc analysis was performed with the TUKEY test when there was significance between the groups. Pearson's test was used for the correlation of normally distributed numerical parameters, and Spearman's test was used for the correlation of abnormally distributed numerical parameters. Chi-square test was performed while analyzing two or more categorical variables. When significance was detected in the Chi-square test in the analysis of more than two categorical variables, post-hoc analysis was performed to determine where the significance originated, and Bonferroni correction was applied to determine the p-value. 'Considering the type 1 error as 0.05 and power as 0.80 in the study, the minimum number of patients included in the study was calculated as 73.'

RESULTS

Out of 82 patients who participated in the study, 44 (53.7%) were female. The mean age was 76.79±6.41 years. 14 patients (17.1%) were university graduates. The most common comorbidities were hypertension (61%), incontinence (40.2%) and diabetes mellitus (29.3%). When grouped as control, MCI, and AD, a significant decrease was observed in the Katz ADL (p<0.001), Lawton IADL (p0.001), MNA-sf (p0.001), MMSE (p0.001) scores. SDC-1 levels were 154.88±22.85 in the control group, 157.95±19.45 in the MCI group, and 159.54±14.04 ng/mL in the AD group. Although there was an increase in SDC-1 level from the control group to the AD group among the groups, no significance was observed (p=0.677). It is shown in detail in Table 1. A box plot graph showing the distribution of serum SDC-1 levels of the patient groups is presented in Figure 1.

When correlation analyzes were performed with SDC-1, a negative correlation was found with Yesavage GDS (Spearman rho: -0.223 p=0.044). No significant correlation was observed with age, ADL, IADL, MMSE score, MNA-SF score (Table 2). When the patients were evaluated according to the Yesavage GDS and grouped as below 5 points and above, the SDC-1 level was 150.543±15.79 ng/mL in depressed patients, and 159.863±18.61 ng/mL in non-depressed patients, and it was significant (p=0.042).

Table 1. Demographic characteristics and clinical data of patients

	Total (n:82)	Control (n:20)	MCI (n:30)	AD (n:32)	p
Female gender, n (%)	44 (53.7)	9 (20.5%)	17 (38.6%)	18 (40.9)	0.671
Age, year	76.79±6.41	72.15±5.38	76.93±6.24	79.56±5.61	**0.017**
Educational level, university graduate, n (%)	14 (17.1)	5 (35.7)	4 (28.6)	5 (35.7)	0.032
Diabetes mellitus, n (%)	24 (29.3)	7 (29.2)	8 (33)	9 (37.5)	0.804
Hypertension, n (%)	50 (61)	13 (26)	20 (40)	17 (34.0)	0.503
Coronary artery disease, n (%)	19 (23.2)	3 (15.7)	7 (36.8)	9 (47.4)	0.551
Incontinence, n (%)	33 (40.2)	6 (18.2)	7 (21.2)	20 (60.6)	**0.004**
Syndecan-1 level, ng/mL	157.81±18.35	154.88±22.85	157.95±19.45	159.54±14.04	0.677
Katz ADL score (min-max)	6 (0-6)	6 (5-6)	6 (5-6)	4 (0-6)	**<0.001**
Lawton-Brody IADL score (min-max)	8 (1-8)	8 (6-8)	8 (4-8)	4 (1-8)	**<0.001**
MNA-SF score (min-max)	13 (1-14)	14 (12-14)	14 (10-14)	11 (1-14)	**<0.001**
Yesavage GDS score (min-max)	2 (0-10)	2 (0-10)	2 (0-10)	2 (0-9)	0.582
MMSE score (min-max)	26 (3-30)	30 (25-30)	27 (18-30)	20 (3-29)	**<0.001**

* SD, standard deviation; MMSE, Mini-Mental State Examination; ADL, Katz Activity of Daily Living; IADL, Lawton Instrumental Activities of Daily Living; MNA-SF, short-form Mini-Nutritional Assessment; GDS, Geriatric Depression Scale.

Table 2. Correlation analysis results of Syndecan-1

	Syndecan-1 level and the correlation coefficient	p
Age	0.05	0.66
ADL	0.096	0.40
IADL	0.035	0.754
MMSE score	0.072	0.521
MNA-SF score	-0.032	0.773
Yesavage GDS score	-0.223	**0.044**

* ADL: Activities of Daily Living, IADL: Instrumental Activities of Daily Living, MMSE: Mini-Mental State Examination, MNA-sf: Mini Nutritional Assessment short-form, GDS: Geriatric Depression Scale.

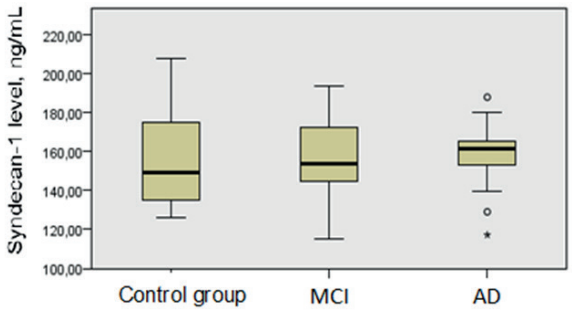

Figure 1. Box plot graph showing the distribution of serum syndecan-1 levels of the patient groups. Values of serum syndecan-1 levels were 154.88±22.85, 157.95±19.45, and 159.54±14.04 ng/mL for the control, MCI, and AD groups, respectively (0.677). MCI: mild cognitive impairment, AD: Alzheimer disease.

DISCUSSION

In this study, the relationship between serum SDC-1 levels and AD were investigated. SDC-1 levels were not different between patients with normal cognitive function, MCI, and AD. While a significant correlation was observed between serum SDC-1 level and Yesavage GDS score, no significant correlation was found with MMSE scores. To our knowledge, this is the first study to analyze serum SDC-1 levels in patients with AD, MCI and normal cognitive function. Although SDC-1 levels had a tendency to increase with cognitive dysfunction, this result was not statistically significant. Therefore, SDC-1 failed to be used as a potential biomarker for AD.

SDCs are the transmembrane HSPG family. SDCs contain a short cytoplasmic domain, a transmembrane domain, and an extracellular domain with additional binding sites for three to five heparan sulfate (HS) or chondroitin sulfate (CS) chains. The HS chains of SDCs enable interaction with many extracellular ligands, while their cytoplasmic domains facilitate intracellular signaling cascades [17]. Among SDCs, SDC-1 has been previously reported to increase cellular uptake of ApoE-containing lipoproteins, thereby facilitating hepatic lipoprotein clearance. Besides the liver, it shows the highest expression of ApoE in the central nervous system. ApoE is also associated with AD according to its isoform in the brain. ApoE2 reduces the risk of AD, while ApoE4 increases it 4 to 14 times [18]. It has been suggested that ApoE is involved in the plaque formation mechanism by directly interacting with amyloid-beta (Aβ) or reducing Aβ clearance [19]. A recent study has presented findings supporting the interaction of ApoEs with HSPGs and the effects of ApoEs on Aβ1-42 uptake and aggregation. On the other hand, it has provided new insights into the molecular interaction of ApoEs with SDCs, a family of transmembrane proteoglycans whose importance is emerging in the pathomechanism of neurodegeneration. In this study, since SDC-3 plays a dominant role in the SDC family, the weak effect of SDC-1 in these mechanisms was also mentioned [20]. In our study, although the SDC-1 level showed an increasing trend from normal cognitive function to AD, no significant relationship was found. This may be attributed to the small number of patients included in the study.

As blood is more accessible than CSF, measuring AD biomarkers is preferred when it comes to sampling for diagnosis and screening. However, developing blood biomarkers for AD has been proven difficult. While CSF is continuous with brain extracellular fluid by free molecule exchange from the brain to CSF, only a part of brain proteins enters blood circulation. Brain proteins released into the blood can be broken down by proteases, metabolized in the liver, or cleared by the kidneys, resulting in a difficult-to-measure condition. For these reasons, the potential to find blood biomarkers for AD is limited [21]. Nevertheless, technical advances in ultrasensitive immunoassays and mass spectrometry have brought new hopes for biomarker discovery [22].

Although amyloid plaques have been defined as primary pathological lesions in AD, studies are still ongoing on how these plaques form in the brain. Increasing evidence suggests that angiogenic vascular factors may play a role in the pathogenic mechanism of AD [23]. Heparan sulfate proteoglycan SDC-1 modulates cell proliferation, adhesion, migration, and angiogenesis. Many studies have shown that SDC-1 may be associated with a number of diseases [6]. The mechanism that could explain the relationship between serum SDC-1 levels and depression is unknown. As a possible mechanism, cortisol levels increase in depression. It is known that depression in advanced age is associated with increased activity of the hypothalamic-pituitary-adrenal (HPA) axis [24]. Considering the studies on SDC-1 and cortisol, it was seen that cortisol suppressed the SDC-1 concentration [25]. In the light of these studies, it is suggested that serum levels may be low in the depression group due to the inhibitory effect of high cortisol on SDC-1 production. In our study, a negative correlation was found between SDC-1 and Yesavage GDS. When the patients were evaluated according to the Yesavage GDS and grouped as below 5 points and above, the SDC-1 level was found to be significantly lower in the patients who scored 6 points and above. There is no article in the literature examining the relationship between SDC-1 and depression.

This study has several limitations. The study was designed as cross-sectional and it has a small sample size. It is thought that the SDC-1 level, which gradually increased from the control group to the AH group, did not create significance due to the small sample size.

It is thought that future therapeutics should be applied in the preclinical and MCI stages of the AD course to maintain the current status. Clarifying the pathogenetic processes more clearly will guide the development of new treatment strategies. This study is important for studying a novel factor as a possible pathogenetic mechanism. However, probably due to small sample size it failed to prove the hypothesis. More extensive studies are needed on this subject.

Author contribution

Study conception and design: RTD, BBD, MH, and MC; data collection: RTD, CC, GSA, CO, HC, and HDV; analysis and interpretation of results: RTD, CC, HDV, and ZGD; draft manuscript preparation: RTD and BBD. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Hacettepe University Ethics Committee (Protocol no. GO 17/963/16.01.2018).

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Conflict of interest

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ORIGINAL ARTICLE

The Accuracy of Provocative Tests on Diabetic Patients with Suspected Carpal Tunnel Syndome and Comparison with Nondiabetics

Çiğdem Tura Bahadır[1]
ORCID: 0000-0001-6492-3064

Sinan Bahadır[2]
ORCID: 0000-0002-1037-5645

[1]Department of Endocrinology and Metabolism, Amasya University Faculty of Medicine, Amasya, Turkey.

[2]Department of Neurosurgery, Amasya University Faculty of Medicine, Amasya, Turkey.

Corresponding Author: Sinan Bahadır
Department of Neurosurgery, Amasya University Faculty of Medicine, Amasya, Turkey.
E-mail: sinanbahadir@windowslive.com

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ABSTRACT

Objective: Carpal tunnel syndrome in diabetic patients differ in some aspects from those in the nondiabetic population. The study was designed to evaluate the diagnostic accuracy of widely used provocative tests in the diabetic population in comparison to nondiabetic population.

Materials and Methods: 87 nondiabetic and 25 diabetic hands suspicious of carpal tunnel syndrome were included in this retrospective study. The presence of carpal tunnel syndrome is confirmed by nerve conduction studies. The hands were divided into DM- and DM+ groups based on patients' diabetes mellitus history. From patient records, results of Tinel's, Phalen's, Durkan's, median nerve compression, and scratch collapse tests were obtained. Sensitivity, specificity, positive predictive value, and negative predictive values of tests are calculated for each population and then compared with each other.

Results: Tinel's test had a higher sensitivity in the diabetic population. Accuracies of Phalen's, Durkan's, median nerve compression, and scratch collapse tests in diabetic patients were similar to those in diabetic patients. None of the tests had a high enough sensitivity to be used alone in either group. Scratch collapse test had very high specificity in both groups but very low sensitivity.

Conclusion: The studied provocative tests have comparable accuracy for carpal tunnel syndrome in diabetic patients to those in nondiabetic patients, with Tinel even having higher sensitivity. But excluding scratch collapse test, none of the tests is strong enough to achieve a diagnosis and none is sensitive enough to rule out a disease.

Key words: carpal tunnel syndrome, diabetes mellitus, provocative tests

INTRODUCTION

Carpal tunnel syndrome (CTS) is the most frequently seen nerve entrapment with a prevalence of 2-4% in the general population [1]. Diabetes mellitus (DM) is an important risk factor for CTS and the prevalence of CTS in the diabetic population varies between 15-30% [2].

CTS is primarily a clinical syndrome. Diagnosis can be based on clinical presentation, physical and neurological examination, provocative tests, electrodiagnostic studies, magnetic resonance imaging, and ultrasonography [3]. In the past, it was observed that some patients with negative electrodiagnostic study results showed improvement after surgery [4]. This finding led to a need for tests that are applicable in the clinical setting and eventually several provocative tests have been developed [4]. The most commonly used provocative tests in the diagnosis of CTS are Tinel's and Phalen's tests [3]. Besides these, Durkan's test, median nerve compression test (MNCT), and relatively novel scratch collapse test (SCT) are among the provocative tests used for CTS diagnosis [5-7]. Though positive nerve conduction studies (NCS) are regarded as the most objective diagnostic criteria, provocative tests are still in use as part of patient evaluation [8]. Moreover, due to the possibility of false-negative results in electrodiagnostic studies, some physicians rely solely upon provocative tests for the diagnosis [9]. And finally, these tests can be used by general practitioners to refer suspected cases to a specialist.

Since diabetes mellitus is a significant risk factor for CTS, there are several studies on diabetic patients with CTS. It has been shown that CTS in the diabetic population may be different than those in the nondiabetic population in some aspects [10-13]. Regarding demographics, Zyluk and Puchalski found that the involvement of older age people, men, and bilateral hands was higher in diabetic CTS [10]. Regarding pathophysiological properties, Tekin et al found that synovial edema, vascular proliferation, and increased wall thickness were more common in diabetic CTS patients [11]. For electrodiagnostic studies, Tsai et al showed that there were significant differences in distal sensory latency, amplitude, and sensory nerve conduction velocity between diabetic and nondiabetic CTS patients [12]. Regarding the response to surgery, Özer et al demonstrated that diabetic patients require a greater improvement in Boston Carpal Tunnel Questionnaire (BCTQ) scores to be satisfied [13].

There is a vast amount of studies on idiopathic CTS in the literature. However, to the best of our knowledge, despite potential differences between diabetic and nondiabetic population, there isn't a study that evaluated the accuracy of provocative tests in the diabetic population.

In this study, the primary aim was to evaluate the accuracy of provocative tests in the clinical assessment of CTS in the symptomatic diabetic patients. Secondary aim was to compare the findings with those in nondiabetic patients.

MATERIALS AND METHODS

A retrospective study of consecutive patients referred to a single neurosurgeon for suspected CTS from November 2020 to November 2021 was performed upon approval by the institutional review board (Amasya University Ethical Commitee of Non-Invasive Clinical Research, Date: 07.10.2021, No: 143). Because of the study's retrospective nature, requirement for informed consent is waived.

The patients were extracted from hospital records with a preliminary diagnosis of CTS. The inclusion criteria were: hands with characteristic symptoms for CTS (paresthesia, pain, weakness, or clumsiness at the distribution site of median nerve; aggravation of symptoms by sleep, repeated movements of hands or wrist, prolonged fixed position; relief of symptoms by shaking hands or position change) and evaluation by provocative tests and electrodiagnostic studies. The exclusion criteria were: age younger than 18 years old, less than 1 month of symptom duration, previous history of fracture, laceration, or operation in the symptomatic hand, cervical radiculopathy, inflammatory joint disease, renal insufficiency, thyroid function disorders, pregnancy, and missing demographic, clinical and/or examination data.

In Tinel's test, the distal wrist crease is tapped 4-5 times and the onset of symptoms is considered positive [14]. In Phalen's test, the wrist is held in palmar flexion while the elbow is extended and considered positive if the symptoms appear

within one minute [15]. For Durkan's test, the examiner applies moderate compression with his/her two thumbs on the flexor retinaculum of the symptomatic hand for 30 seconds [5]. MNCT was performed while the elbow is extended, the forearm is supinated, and the wrist is flexed at 60° [6]. With his/her thumb, the examiner applies pressure on the carpal tunnel [6]. In SCT test, with elbows in 90° flexion and forearms in 90° pronation, the patient resists with bilateral shoulder external rotation to the force applied on the lateral side of forearms [7]. In the presence of allodynia secondary to nerve entrapment, a temporary decrease in muscle resistance occurs following a gentle swipe of the nerve entrapment area with the examiner's fingers, and the test is considered positive [7].

Demographic data and past disease history (including laboratory and imaging studies when necessary) of the patients; and presenting symptoms, examination findings, and NCS results of symptomatic hands were extracted from the hospital's patient database and were recorded. The hands were divided into DM- (non-diabetic) and DM+ (diabetic) groups based on the absence or presence of DM, respectively. Then the groups were subdivided into two subgroups as CTS- and CTS+ based on the absence or presence of CTS based on electrodiagnostic studies.

Statistical Analysis

Continuous data are expressed as mean ± standard deviation, categorical data are expressed as count and frequency. Continuous data were evaluated either by unpaired t-test or Mann-Whitney U test based on the distribution of data which was analyzed by the Shapiro-Wilk test.

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of evaluated provocative tests were calculated for DM- and DM+ groups separately. Intergroup comparison was performed by Fisher's exact test. $P<0.05$ was considered statistically significant.

RESULTS

The retrospective data search yielded 183 hands (110 patients) that were evaluated by electrodiagnostic studies for preliminary diagnosis of CTS. 54 hands were excluded for thyroid function disorders, extremity and peripheral nerve pathologies, and compressive cervical disc herniation. Demographic data of 80 patients and 129 hands that were included in the study are summarized in Table 1. Diabetic patients were older and have a higher body mass index (BMI) compared to nondiabetic patients, but symptom duration did not differ between the two groups. The incidence of bilateral symptoms was also similar in both groups but electrodiagnostically confirmed bilateral CTS was significantly more frequent in diabetic patients. CTS was confirmed electrodiagnostically in all but two hands (95.2%) in the DM+ group whereas this rate was lower (73.6%) in nondiabetic hands (P=0.040).

Provocative tests used in the clinical setting and thus evaluated in this study were Tinel's test, Phalen's test, MNCT, Durkan's test, and SCT. The sensitivity, specificity, PPV, and NPV of tests were summarized in Table 2.

The most sensitive test in both groups was Phalen's test with no significant difference between groups (70.5% for DM-, 76.3% for DM+). The least sensitive test was SCT in both groups. Regarding sensitivity, only Tinel's test was different between groups (P=0.035), however, it was the second least sensitive test.

SCT was the most specific test in both groups (95.7% for DM-, 100.0% for DM+). No test varied significantly regarding specificity between the two groups. The second most specific test in DM- was Tinel's test (73.9%).

Both in DM- and DM+ groups, SCT had the highest PPV (92.3% and 100.0%, respectively). Excluding SCT, the overall highest PPV in DM- group was 78.3% (Durkan's test) while the overall smallest PPV in the DM+ group was 86.7% (Durkan's test). Still, only MNCT showed a significant difference between DM- and DM+ groups (75.6% vs 95.8%, respectively; P=0.457).

Overall NPVs were very low in both groups. The highest NPVs were achieved with Phalen's test (33.3%) and SCT (5.7%) in DM- and DM+ groups, respectively.

DISCUSSION

The study revealed a few important findings. None of the provocative tests had a significantly worse sensitivity in the diabetic group compared

Table 1. Demographic data of patients and symptomatic hands with and without diabetes mellitus.

	Nondiabetic patients (n=55)	Diabetic patients (n=25)	P
Gender (M:F)	15:40	3:22	0.158
Age (years) (median, range)	47 (28-76)	56 (19-72)	0.002*
BMI (median, range)	27.43 (21.29-47.27)	35.11 (24.14-47.66)	0.001*
Symptomatic hands (n,%)	87 (79.0%)	42 (84.0%)	0.524
Bilateral symptoms (n)	32	17	0.465
Confirmed bilateral CTS	21/32	16/17	0.037*
Duration of diabetes mellitus (median, range)	Not applicable	10 years (2 years – 22 years)	
Type of diabetes (Type I:Type II)	Not applicable	1:24	
Diabetic neuropathy (n)	Not applicable	7	
Diabetic vasculopathy (n)	Not applicable	2	
Diabetic nephropathy (n)	Not applicable	1	
	Nondiabetic hands (n=87)	Diabetic hands (42)	
Confirmed CTS (n,%)	64 (73.6%)	40 (95.2%)	0.040*
Symptom duration (months) (median, range)	12 (1-240)	12 (1-240)	0.479
Motor weakness (n,%)	9 (10.3%)	5 (11.9%)	0.770
Sensory disturbance (n,%)	29 (33.3%)	15 (35.7%)	0.844
Tenar atrophy (n,%)	7 (8%)	21 (50%)	0.095
Number of Tinel's test performed (n)	87	42	
Number of Phalen's test performed (n)	82	40	
Number of Durkan's test performed (n)	48	23	
Number of MNCT performed (n)	86	42	
Number of SCT performed (n)	87	40	

* Statistically significant
M: male, F: female, BMI: body mass index, CTS: carpal tunnel syndrome, MNCT: median nerve compression test, SCT: scratch collapse test

Table 2. Sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) of the tests when used alone.

	Sensitivity (%)			Specificity (%)			PPV (%)			NPV (%)		
	DM-	DM+	P	DM-	DM+	P	DM-	DM+	P	DM-	DM+	P
Tinel	26.6	47.5	0.035*	73.9	50.0	0.490	73.9	95.0	0.100	26.6	4.5	0.033*
Phalen	70.5	76.3	0.644	42.9	0.0	0.502	78.2	93.5	0.075	33.3	0.0	0.076
Durkan	51.4	61.9	0.580	61.5	0.0	0.200	78.3	86.7	0.681	32.0	0.0	0.152
MNCT	54.0	57.5	0.839	52.2	50.0	1	75.6	95.8	0.046*	29.3	5.6	0.049*
SCT	18.8	13.2	0.587	95.7	100.0	1	92.3	100.0	1	29.7	5.7	0.005*

* Statistically significant
MNCT: median nevre compression test, SCT: scratch collapse test

to nondiabetic group. In fact, Tinel's test had a significantly higher sensitivity in the diabetic group. Regarding specificity, two groups didn't differ in any provocative tests. SCT had the highest specificity in both groups. One important issue is almost all of the symptomatic hands in diabetic patients had electrodiagnostically confirmed CST, which is close to PPV of provocative tests, excluding SCT. The most sensitive test was Phalen's test in both groups. Durkan and MNCT did not excel in any parameter in any group and are not the best candidates to be used as standalone tests. The sensitivity of SCT was very low in both groups compared to the literature. However, it had a very high specificity and PPV.

CTS is principally a clinical syndrome where an asymptomatic patient cannot be diagnosed with the disease despite having a positive NCS, which should be considered as median neuropathy at the wrist [9]. Though NCS is regarded as the most reliable diagnostic tool for CTS, provocative tests have still been in use for clinical evaluation [3].

The accuracy of provocative tests in idiopathic CTS has been widely studied in the literature [4, 6, 7, 16-23]. When the diagnosis is based on electrodiagnostic studies, sensitivity, specificity, PPV, and NPVs of these tests show great variation across different reports [4, 6, 7, 16-23]. Such studies either excluded diabetic patients, or didn't look for DM at all, or only evaluated those with neuropathy. So, the accuracy of provocative tests in diabetic patients remained unclear.

One of the oldest and most widely used provocative tests is Tinel's test [14]. Tinel's sign is defined as a phenomenon observed during the regeneration following demyelination in peripheral nerve injuries [18, 24]. In their prospective study which didn't exclude any diabetic patients, Tetro et al compared Tinel's, Phalen's, Durkan's, and MNCT tests and found a sensitivity and specificty values of 75% and 91%, respectively, for Tinel's test [6]. They also hypothetically found Tinel's test's PPV and NPV ranging between 29-89% and 78-99%, respectively [6]. In Mondelli et al's and El Miedany et al's studies which both excluded diabetic patients, Tinel's sensitivity (30% and 41%, respectively) was much lower than Tetro et al's findings, but specificity (65% and 90%, respectively) was similar [18, 20]. In a recent study by Kasundra et al which included diabetic patients, Tinel's test had a sensitivity and specificity of 78.5% and 91.3%, respectively [3]. In another prospective study by Küçükakkaş et al, which also included diabetic patients, Tinel's test showed a sensitivity of 89%, a specificity of 41%, a PPV of 59%, and an NPV of 80% [25]. This variation in sensitivity of Tinel's test might have resulted from inclusion of diabetic patients. But in 2020, in a prospective study conducted on symptomatic patients that included diabetic patients, Zhang et al found Tinel's test's sensitivity as 47%, specificity as 56%, PPV as 90%, and NPV as 11% [23]. Tinel's sensitivity in our study was also higher in diabetic hands which can be attributed to ongoing neuronal injury in diabetic patients.

The purpose in Phalen's test is to provoke symptoms by inducing ischemia with compression of the median nerve which is already under compression and has a lower threshold for mechanical pressure [17]. Tetro et al found a lower sensitivity and specificity values (61% and 83%, respectively) for Phalen's test compared to Tinel's test [6]. They found PPV and NPV for Phalen's test ranging between 16-79% and 68-98%, respectively, depending on the prevalence of the disease [6]. Mondelli et al and El Miedany et al found similar, albeit slightly lower, sensitivity values for Phalen's test (59% and 47%, respectively) compared to Tetro et al's study, but specificity values varied greatly (93% and 17%, respectively) [18, 20]. Zhang et al found Phalen's test had a sensitivity of 50%, specificity of 33%, PPV of 86%, and NPV of 7% [23]. Kasundra et al found higher sensitivity (84.9%) and specificity (73.9%) values for Phalen's test compared to previous studies [3]. Küçükakkaş et al's findings, showed similar sensitivity, specificity, PPV, and NPV for Phalen's test (86%, 57%, 66%, and 81%, respectively) to those in previous reports [25]. Similarly, in our study, Phalen's test showed no significant difference between diabetic and nondiabetic hands.

Durkan's test has a similar mechanism to Phalen's test. Its sensitivity and specificty were found as 75% and 93%, respectively, by Tetro et al [6]. Its PPV ranged between 35-91%, and NPV ranged between 79-99% [6]. In their prospective study where diabetic patients were not excluded and only symptomatic patients were included, Kaul et al found sensitivitity and specificity of Durkan's test as 52.5% and 61.8% respectively, much lower than Tetro et al's findings [17]. PPV and NPV of Durkan's test were 66.6% and 47.2%, respectively, according to same study [17]. Küçükakkaş et al found sensitivity of 96% and a specificity of 67% for Durkan's test [25]. They found similar PPV (73%) and NPV (94%) to Tetro et al's findings [25]. In Zhang et al's study, Durkan's test had a sensitivity of 71%, similar to literature; specificity of 22% that is very low compared to previous studies; PPV of 89%; and NPV of 8% [23].

MNCT attempts to combine Phalen's and Durkan's tests in one test. Tetro et al found MNCT to be more sensitive (86%) than both Tinel's, Phalen's and Durkan's tests, but only more specific (95%) than Phalen's test [6]. It also had a higher PPV and NPV (94% and 87%, respectively) than Phalen's test when the hypothetical prevalence rate was 0.5 [6]. On the other hand, Cheng et al, in their prospective study, reported a much lower sensitivity (44%), but a similar specificity (99%) for the test [7]. Zhang et al found a similar sensitivity (84%) to Tetro et al's findings accompanied with a very low specificity 11% [23]. They also found a PPV of 89% and NPV of 8% [23].

In the relatively novel SCT test, the exact mechanism is unknown, it is thought to be related to the cutaneous silent period where inhibitory spinal reflexes play a role [7]. Cheng et al initially a sensitivity of 64% and a specificity of 99% for SCT in their paper where they introduced the test [7]. They also found a PPV of 99% and a MPV of 82% fort he test [7]. They found the test significantly more sensitive than both Tinel's test and MNCT [7]. In a prospective study by Simon et al, in which diabetic patients were not excluded, SCT had a sensitivity of 28%, specificity of 75%, PPV of 81%, and NPV of 20% [22].

Such a variety among studies regarding provocative tests -including ours- may arise from study design, selected population, measurement and evaluation methods, and statistical methods [18]. A high level of sensitivity in a population consisting of patients with severe and classical symptoms would not be achievable in a population that includes less typical cases [17]. On the other hand, when the control group is composed of healthy subjects, there can be specificity bias [17]. In a study by Gerr et al [26], the specificity of Phalen's test was 97% when the control group consisted of healthy subjects, however, it dropped to 61% when the control group is composed of patients with symptoms but didn't have CTS. Similarly, Descatha et al [27] found that provocative tests were not effective screening tools in patients that don't have complaints severe enough to seek healthcare. For these reasons, it is essential to interpret the findings of provocative test studies with the study population in mind.

Though some studies included diabetic patients in their cohorts while evaluating the accuracy of provocative tests, none had compared the findings between these two group of patients. The findings of this study showed that some tests's accuracy may differ between these patients.

The study is not deprived of limitations: First, since the study is retrospective, blindness could not be achieved. However, apart from SCT, all the tests depend on patients's responses and examiner's bias hardly affects the results. Second, there might have been selection bias since only symptomatic hands were evaluated. However, since these tests aren't screening tests for healthy subjects but tests that are performed on patients with complaints, we think that this type of study population conforms to clinical practice better. Third, since both symptomatic hands were included in the study, the samples are not completely independent. Finally, the diabetic group had a relatively low number of samples compared to non-diabetic group. Besides, conditions which are frequently seen in the diabetic population like neuropathy, hypertension, and hyperlipidemia, or disease related conditions such as blood glucose control status, treatment type, or type of DM might have impaired the homogeneity of this group. Also, it must be noted that in the diabetic group, only 2 hands turned out to be CTS negative and this low value might have affected the reliability and generalizability of specificity and NPV of tests in this group.

CONCLUSION

Provocative tests in diabetic patients are as accurate as in the nondiabetic population. Excluding SCT which has a very high specificity, none of the tests are sensitive and specific enough to be used alone for the clinical diagnosis of CTS, regardless of diabetes mellitus. In populations similar to those of the study, SCT can be used as a diagnostic tool. On the other hand, no negative results of these tests can rule out the disease.

Author contribution

Study conception and design: ÇTB and SB; data collection: ÇTB and SB; analysis and interpretation of results: SB; draft manuscript preparation: ÇTB and SB. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Amasya University Ethical Commitee of Non-Invasive Clinical Research (Protocol no: 143/Date: 07.10.2021).

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Conflict of interest

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acta medica

ORIGINAL ARTICLE

Biliary Tract Disorders in Patients with Acromegaly: Single-centre Experience

Süleyman Nahit Şendur[1]
ORCID: 0000-0001-6740-6708

Seda Hanife Oğuz[1]
ORCID: 0000-0002-7781-944X

[1]Hacettepe University, Faculty of Medicine, Department of Endocrinology & Metabolism, Ankara, Turkey.

Corresponding Author: Süleyman Nahit Şendur
Hacettepe University, Faculty of Medicine, Department of Endocrinology & Metabolism, Ankara, Turkey.
E-mail: snahitsendur@hotmail.com

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ABSTRACT

Objective: Genetic and environmental factors determine the risk of biliary tract disorders. Several studies have reported an increased frequency of gallbladder disorders in patients with acromegaly belonging to different ethnic groups, however, data for Turkish patients with acromegaly is currently lacking. The primary objective of this study was to evaluate the frequency of diverse gallbladder disorders in Turkish patients with acromegaly. The secondary objective was to determine the risk factors that are related to gallstone formation.

Materials and Methods: Hacettepe University electronic database was retrospectively analyzed. One hundred fifty-two out of 393 patients with acromegaly who had confirmed biliary tract disorders with imaging modalities (such as abdominal ultrasonography, abdominal computed tomography or abdominal magnetic resonance imaging) or surgery specimens, were included for final analyzes. There was not any difference between included and excluded cases in terms of study parameters.

Results: Patients with acromegaly with a mean±SD age of 41±12 years were followed-up for median of 91 months. Gender distribution was equal (76 M, 76 F). Gallstones were detected in 50 (33%) cases. Seventeen (11%) cases had gallbladder sludge whereas 5 (3%) cases had gallbladder polyp. Cholecystectomy was performed in 24 (16%). Age, gender, baseline disease activity, diagnostic delay, disease duration, the presence of type 2 diabetes mellitus and hyperlipidemia were not associated with gallstone formation. Body mass index (β=1.19, 95% CI (1.09-1.30), p<0.001) and somatostatin receptor ligand use (β=3.8, 95% CI (1.2-12.6), p=0.026) were determined as independent risk factors for cholelithiasis.

Conclusions: Biliary tract disorders are common in Turkish patients with acromegaly. Acromegaly patients with high body mass index and on somatostatin receptor ligand treatment had an increased risk for gallstone disease.

Keywords: Acromegaly, gallbladder, cholecystolithiasis, cholelithiasis, polyp, sludge, somatostatin receptor ligand

INTRODUCTION

Acromegaly is a unique disorder characterized by chronic growth hormone (GH) and insulin-like growth factor-1 (IGF-1) hypersecretion. Over 95% of the cases a GH-secreting pituitary adenoma arising from somatotroph cells causes GH overproduction; rarely, growth hormone-releasing hormone secretion from a neuroendocrine tumor or more rarely, ectopic GH release by an abdominal or hematopoietic tumor may result in acromegaly [1].

Chronic exposure to excess GH and IGF-1 leads to systemic manifestations and, gastrointestinal and hepatobiliary disorders are frequently encountered in patients with acromegaly [2]. For instance, bowel length increases up to 20% [3]. Colonic diverticulosis is common; hyperplastic and adenomatous polyps could be seen and both are associated with disease activation [4,5]. Colorectal cancer risk has been shown to be higher [6]. There is an increased prevalence of gallbladder polyps in patients with acromegaly and the risk is higher in patients with higher GH levels [7]. Not disease activity per se however, somatostatin receptor ligands (SRLs) which are the mainstay of the medical management of acromegaly, induce gallstone and sludge formation and, several studies have reported increased frequency of gallstone disease with varying proportions in patients with acromegaly belonging to different ethnicities [8-10].

In this study, we aimed to evaluate the frequency of biliary tract disorders and the predictive factors for the biliary tract stone onset in a large population of Turkish patients with acromegaly.

MATERIALS AND METHODS

Study design, study population and study parameters

Data of 393 patients with acromegaly who were followed up between 1980 and 2018 at the Department of Endocrinology and Metabolism, Hacettepe University, Ankara, Turkey were retrospectively analyzed. As shown in the inclusion diagram (Figure 1), final analyzes were performed in 152 patients with biliary disorders that were confirmed with imaging modalities such as hepatobiliary ultrasonography, abdominal computed tomography, abdominal magnetic resonance imaging and/or surgery specimens. There were no differences between included and excluded cases in terms of study parameters.

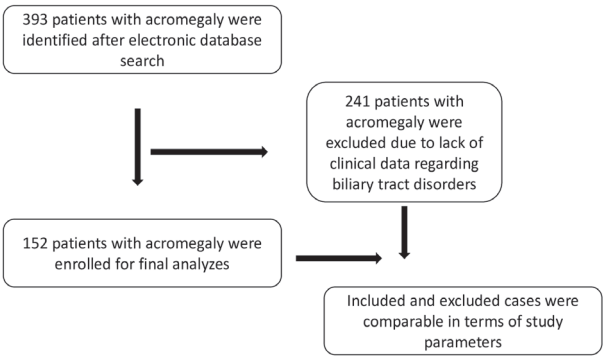

Figure 1. Inclusion diagram

For each patient, the following data were recorded: age at the diagnosis of acromegaly (years), age at the first symptoms related to acromegaly (years), gender, time from symptoms to diagnosis (years), disease duration (years), follow-up duration (months), height (cm), weight (kg), body mass index (BMI) (kg/m2), GH level at diagnosis (ng/mL), IGF-1 level at diagnosis (ng/mL), adenoma size (mm), first-line treatment for acromegaly, medications for acromegaly, presence of type 2 diabetes mellitus and hyperlipidemia. Based on the imaging and surgery data, information relative to gallstones, sludge, gallbladder polyps and cholecystectomy was collected.

The study was conducted in accordance with guidelines in the Declaration of Helsinki and its later amendments. Hacettepe University Ethical Board approved the study with the project number GO 19/303.

Statistical analysis

All analyzes were performed with Statistical Package for Social Sciences (SPSS) version 21.0. The distributions of continuous variables were tested for normality by using visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk's test). Descriptive measures of variables with normal distribution were presented as the mean ± standard deviation. Median-interquartile range (IQR) was used to describe continuous variables with a skewed distribution. Categorical variables

were expressed as frequencies and/or percentages where appropriate. Student's t-test and Mann-Whitney U test were used to compare differences in normally distributed and non-normally distributed continuous variables, respectively. The differences in categorical variables were assessed by The Chi-square or Fisher's exact test. While investigating the associations between variables, the correlation coefficients and their significance were calculated using the Pearson or the Spearman tests. For the multivariate analysis, the possible factors identified with univariate analysis were further entered into the regression analysis to determine independent predictors of the presence of cholecystolithiasis. The model fit was assessed using appropriate residual and goodness-of-fit statistics. A 5% type-1 error level was used to infer statistical significance. A p-value ≤ 0.05 was accepted as statistically significant.

RESULTS

Demographic, clinical and biochemical characteristics of patients with acromegaly

Demographic, clinical and biochemical characteristics of patients with acromegaly were summarized in Table 1.

Accordingly, the mean age at the diagnosis of patients with acromegaly was 41±12 years. Diagnostic delay was median (IQR) of 2 (0-5) years from the onset of the first symptoms. Patients with acromegaly had a disease duration of 17±10 years and the median (IQR) follow-up length was 91 (37-164) months. Gender distribution was equal (76 male and 76 female) and the patients with acromegaly were mostly overweight or slightly obese; mean±SD BMI 31.9±7.6 kg/m2. GH and IGF-1 levels at diagnosis were 10.4 (5.0-30.4) ng/mL and 859 (608-1079) ng/mL, respectively. All patients had pituitary adenomas as the culprit lesions of excess GH secretion. The size of pituitary adenoma was available in 132 patients and in the vast majority of patients, adenoma was larger than 10 mm; mean±SD adenoma size 18.1±9.2 mm, 82% macroadenoma. Almost in all patients (97%), transsphenoidal surgery was performed as first-line treatment. Three patients were not suitable candidates for surgery due to high surgery risk. Remission could not be achieved with surgery

Table 1. Demographic, clinical and biochemical characteristics of patients with acromegaly, n=152

Parameter	Value
Age at the diagnosis of acromegaly (years)	41±12
Age at the first symptoms related to acromegaly (years)	37±12
Time from symptoms to diagnose (years) *	2 (0-5)
Disease duration (years)	17±10
Follow-up duration (months) *	91 (37-164)
Gender F-M n (%)	76-76 (50-50)
Height (cm)	166±12
Weight (cm)	86±17
Body mass index (kg/m^2)	31.9±7.6
GH level at diagnosis (ng/mL)*	10.4 (5.0-30.4)
IGF-1 level at diagnosis (ng/mL)*	859 (608-1079)
Adenoma size (mm)**	18.1±9.2
Macroadenoma n (%)	100 (82)
Microadenoma n (%)	22 (18)
First-line treatment n (%)	
Surgery	146 (97)
Radiotherapy	0 (0)
Medical treatment	4 (3)
Medical treatment n (%)	
SRL	
Yes	100 (67)
No	49 (33)
Pegvisomant	
Yes	7 (5)
No	141 (95)
Cabergoline	
Yes	28 (19)
No	119 (81)
Type 2 diabetes mellitus n (%)	
Yes	56 (38)
No	91 (62)
Hyperlipidemia n (%)	
Yes	51 (36)
No	92 (64)
Gallbladder disorders n (%)	
Cholecystolithiasis	
Yes	50 (33)
No	102 (67)
Galbladder sludge	
Yes	17 (11)
No	135 (89)
Polyp	
Yes	5 (3)
No	147 (97)
Surgery for gallbladder	
Yes	24 (16)
No	128 (84)

*The data was given as median (interquartile range)

**Adenoma size data was not avaliable in 30 patients

in most of the patients and medical treatment was initiated. While most patients were under SRLs (67%), 5% and 19% of cases were using pegvisomant and cabergoline, respectively. Nearly one in third of patients with acromegaly had type 2 diabetes mellitus (38%) or hyperlipidemia (36%).

Gallstones were detected in 50 (33%) cases and 17 (11%) cases had gallbladder sludge whereas 5 (3%) cases had gallbladder polyps. Cholecystectomy was performed in 24 (16%) patients with acromegaly without any complications.

Determination of risk factors for gallstone disorder in univariate and multivariate analyses

There were not any differences between patients with gallstones and patients without gallstones in terms of age at the diagnosis of acromegaly, age at the first symptoms related to acromegaly, diagnostic delay, disease duration, gender, GH and IGF-1 levels at diagnosis, first-line treatment modalities, the frequency of type 2 diabetes mellitus and the frequency of hyperlipidemia (Table 2).

Patients with cholecystolithiasis were heavier and had a higher BMI; weight (kg), patients with

Table 2. Comparison of risk factors between patients without and with cholecystolithiasis, n=152

Parameter	Patients without gallstone n= 102	Patients with gallstone n= 50	p value
Age at the diagnosis of acromegaly (years)	41.4±11.7	40.1±12.0	0.53
Age at the first symptoms related to acromegaly (years)	37.2±11.6	37.3±12.3	0.95
Time from symptoms to diagnose (years)	3 (0-5)	2 (0-5)	0.41
Disease duration (years)	18.3±9.8	16.9±9.6	0.42
Gender F-M n (%)	48-53 (48-52)	28-23 (55-45)	0.39
Height (cm)	168±12	160±11	**<0.001**
Weight (cm)	82±15	94±17	**<0.001**
Body mass index (kg/m^2)	28.8±5.4	37.3±8.0	**<0.001**
GH level at diagnosis (ng/mL)*	10.1 (5.1-29.5)	10.8 (4.0-34.7)	0.97
IGF-1 level at diagnosis (ng/mL)*	833 (554-1070)	878 (722-1088)	0.38
Adenom size (mm)**	17.0±8.5	20.6±10.3	
Macroadenom n (%)	64 (77)	36 (92)	**0.042**
Microadenom n (%)	19 (23)	3 (8)	
First-line treatment n (%)			
Surgery	97 (97)	49 (98)	0.72
Radiotherapy	0	0	
Medical treatment	3 (3)	1 (2)	
Medical treatment n (%)			
SRL			
Yes	57 (58)	43 (86)	<0.001
No	42 (42)	7 (14)	
Pegvisomant			
Yes	2 (2)	5 (10)	0.031
No	96 (98)	45 (90)	
Cabergoline			
Yes	18 (19)	10 (20)	0.83
No	79 (81)	40 (80)	
Type 2 diabetes mellitus n (%)			
Yes	34 (35)	22 (44)	0.37
No	63 (65)	28 (56)	
Hyperlipidemia n (%)			
Yes	63 (66)	29 (60)	0.58
No	32 (34)	19 (40)	

*The data was given as median (interquartile range)

**Adenoma size data was not avaliable in 30 patients

gallstone 94±17 vs patients without gallstone 82±15, p<0.001; BMI (kg/m2), patients with gallstone 37.3±8.0 vs patients without gallstone 28.8±5.4, p<0.001. Adenomas were larger in patients with gallstone; macroadenoma frequency 92% vs 77%, p= 0.042. Patients were more frequently on SRL or pegvisomant therapy in gallstone group; SRL 86% vs 58%, p<0.001; pegvisomant 10% vs 2%, p=0.031 (Table 2).

Variables determined from univariate analysis were entered into multiple linear regression model to reveal factors that are associated with gallstone formation. As shown in Table 3, among the independent variables, BMI (β=1.19, 95% CI (1.09-1.30), p<0.001) and SRL use (β=3.8, 95% CI (1.2-12.6), p=0.026) were found to be associated with variations in cholecystolithiasis frequency after adjusting for the other co-variables in the model.

Table 3. Risk factors for cholecystolithiasis

Parameter	OR (95%CI)	p value
Somatostatin receptor ligand use	3.8 (1.2-12.6)	0.026
Presence of macroadenoma	1.7 (0.4-7.7)	0.48
Pegvisomant use	4.4 (0.7-28.7)	0.12
Body mass index	1.19 (1.09-1.30)	<0.001

DISCUSSION

The present study has highlighted that biliary tract disorders are frequent in patients with acromegaly. In our cohort, one-third of patients with acromegaly had gallstones and radiologic examinations revealed at least one gallbladder disorder in almost half of the cases. Increased BMI and SRL use were determined as independent risk factors for gallstone formation.

Gallstone disease is common in adult population. The interplay between exogenous factors such as dietary habits and genetic background determines the risk for gallstone formation, therefore the prevalence of gallstone disease varies by geographic region and ethnicity [11]. In Europe, about 20% of the adult population develops gallstones and in the United States, approximately 6% of men and 9% of women have gallstones [12,13]. The risk is highest in Native Americans followed by Hispanic Americans and non-Hispanic Whites. African populations represent the lowest risk group for gallstone disease and the prevalence rates are intermediate in Asian populations varying between 5 to 20% [11,14]. Although there isn't any population-based study assessing the frequency of gallstone disease in the Turkish adult population, Karayalcin et al have found a 15.4% prevalence in a sample of postmenopausal women including 474 females [15]. In our cohort, which included younger individuals and had an equal gender distribution, 33% of patients with acromegaly had gallstone disease. Considering the increased prevalence of gallstone disease in older age and women, it can crudely be said that in our cohort, patients with acromegaly had an increased frequency of gallstone disease when compared to reference Turkish population [11,15].

In addition, gallstones have been variably reported ranging from 3.6 to 56% in patients with acromegaly and in most studies, consistently, the prevalence of gallstone disease has been found to be higher in patients with acromegaly when compared to reference population [9]. However, to date, there is no data regarding the gallstone disease prevalence in Turkish patients with acromegaly. Our study is the first study that evaluates the frequency of gallstone disease in a large cohort of Turkish patients with acromegaly.

Bile is a dark green to yellowish-brown fluid produced by the liver and facilitates the digestion of lipids in intestine. It comprises mainly water (>90%) with bile salts, phospholipids, cholesterol, conjugated bilirubin and electrolytes [16,17]. The generation of cholesterol gallstones is the result of the disruption of cholesterol solubility in bile [18]. Hepatic over-secretion of cholesterol and/or reduced secretion of bile acids and phospholipids lead to an equilibrium that is supersaturated in which bile contains excess amounts of cholesterol that cannot be solubilized by bile salts and phospholipids. Impaired gallbladder emptying, by increasing the residence time of cholesterol-supersaturated bile in the gallbladder lumen, promotes nucleation of cholesterol crystals (sludge) which are the precursors of gallstones. In some cases, prolongation of intestinal transit time contributes to gallstone formation by reducing absorption of bile salts [11].

Hyperinsulinemia induces uptake of cholesterol by hepatocytes and increases biliary secretion of cholesterol [19,20]. Moreover, in the case of

hyperinsulinemia, the excretion of bile acids into the bile is reduced [21]. Consequently, bile is supersaturated and hyperinsulinemic subjects are susceptible to gallstone formation. Due to insulin resistance, patients with obesity have increased insulin levels and obesity is a well-defined risk factor for gallstone disease [22]. Accordingly, patients with obesity are predisposed to gallstone formation, symptomatic gallstones and cholecystectomy [23,24]. In our study, we have found that acromegalic patients with gallstone disease were heavier than their counterparts and for each unit increase in BMI, the risk of cholelithiasis increased ~1.2 times. Thus, obesity is a risk factor for gallstone disease in patients with acromegaly as well.

Transsphenoidal adenoma excision is the first-line treatment in acromegaly but medical treatment is employed in most patients due to inoperable tumors or disease persistence following surgery. SRLs are considered the mainstay in the medical management of acromegaly [1]. These drugs have been used extensively over three decades and overall possess a favorable benefit-risk profile [25]. They are generally well tolerated; the most reported side effects include nausea, diarrhea, abdominal pain and distension. These side effects are self-limited and mainly occur in the first weeks of treatment [26]. It is not surprising that most side effects of these drugs are related to the gastrointestinal system because the hepatobiliary and alimentary tracts are well-defined targets of somatostatin activity [27]. Indeed, SRLs have several actions that may contribute to gallstone formation. They decrease cholecystokinin secretion from small intestine, which is the main stimulator of gallbladder contraction, and inhibit the contractile response of the gallbladder to cholecystokinin [28,29]. By triggering the absorption of sodium and water by the gallbladder, SRLs lead to increase in bile concentration [30]. Moreover, SRLs hinder the physiologic post-prandial relaxation of the Oddi sphincter and promote crystallization and stone formation [31]. Accordingly, several studies have reported an increased prevalence rate of gallstones in patients receiving SRLs either due to acromegaly or other neuroendocrine neoplasms [10,32,33]. Our data confirm that gallstone disease is common in Turkish patients with acromegaly as well and SRL use was the main risk factor for cholelithiasis.

We did not find any associations between the presence of gallstone disease and diagnostic delay, GH level at diagnosis and IGF-1 level at diagnosis. Rather than the activity of acromegaly per se, the most important risk factor for the occurrence of gallstone disease in acromegaly is SRL use which is consistent with previous reports [10,32].

There were particular limitations of our study. Due to the retrospective and cross-sectional design of our study, we could not assess the course of gallbladder disorders and we were not able to collect information regarding the medical treatment of gallstone disease. In addition, there was no information about the status of the gallbladder of the patients before SRL treatment.

In conclusion, gallbladder disorders are frequent in patients with acromegaly. Increased BMI and SRL treatment are the main risk factors for gallstone disease. Clinicians should evaluate the patients with acromegaly, especially the patients who have high BMI and are on SRL treatment, with imaging modalities for early recognition of gallbladder disorders.

Author contribution

Study conception and design: SNŞ and SHO; data collection: SNŞ; analysis and interpretation of results: SNŞ and SHO; draft manuscript preparation: SNŞ and SHO. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Hacettepe University Ethical Board (Protocol no. GO-19/303/19.03.2019).

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Conflict of interest

The authors declare that there is no conflict of interest.

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CASE REPORT

Anesthesia Management in An Adult Patient with Desbuquois Syndrome

Murat Tümer[1]
ORCID: 0000-0001-9132-9992

Hazar Kökten[1]
ORCID: 0000-0001-7146-816X

Aysun Ankay Yılbaş[1]
ORCID: 0000-0001-6196-3191

Fatma Sarıcaoğlu[1]
ORCID: 0000-0002-2706-5651

[1]Department of Anesthesiology and Reanimation, Faculty of Medicine, Hacettepe University, Ankara, Turkey.

Corresponding Author: Murat Tümer
Department of Anesthesiology and Reanimation, Faculty of Medicine, Hacettepe University, Ankara, Turkey.

E-mail: dr.m.tumer@gmail.com

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ABSTRACT

Desbuquois syndrome (DS) is a rare disease that can be accompanied by a difficult airway. In the literature, there are usually case reports about the mortal forms in childhood. We wanted to share our anesthesia experience in an adult patient with DS who underwent orthopedic surgery. We used videolaryngoscopy for intubation with coronavirus precautions. Adult patients with DS can be managed safely with adequate difficult airway preparation.

Keywords: Desbuquois syndrome, difficult airway, anesthesia management

INTRODUCTION

Desbuquois Syndrome (DS) is an autosomal recessive syndrome characterized by short stature, joint dislocation, and defects in the hand and foot bones [1,2]. There are two types of DS, Type-1 (mild form) with a mutation in the CANT-1 gene and Type-2 (severe form) with XYLT1 mutation. Airway management can be challenging due to short neck and facial deformities. In our knowledge, there is only one case report of an infant about airway management of DS in literature [3]. We share our experience with an adult DS patient who underwent femoral derotation osteotomy under general anesthesia.

CASE PRESENTATION

A 20-year-old male patient with DS was consulted for femoral derotation osteotomy. The written consent has been obtained from the patient. The patient's height was 139 cm (<3 percentile), weight was 68.5 kg (50-75 percentile), and head circumference was 51.5 cm (<3 percentile. His body mass index was 36 kg/m^2. He had a short neck, prognathism, depressed nasal ridge (Figure 1). His Mallampati score was III. There were no other signs about a difficult airway at his physical exam. He was taken to the operating room with Covid-19 pandemic precautions.

Figure 1. 20-year-old male patient with Desbuquois Syndrome. His height was under 3 percentiles. He had a short neck, prognathism, depressed nasal ridge. His mallampati score was III.

After routine monitoring, the patient was pre-oxygenated with 100% FiO2 for 5 minutes. Rapid sequence induction was performed with propofol, fentanyl, and rocuronium. After induction, invasive artery and temperature monitoring was performed. Mask ventilation was easy. He was intubated using a 7.5 cuffed endotracheal tube with GlideScope® videolaryngoscope (GVL; Verathon, Bothell, WA, USA). His Cormack-Lehane score was II. Total intravenous anesthesia (propofol and remifentanil) was used for maintenance. After the operation was completed without any problems, muscle relaxation was reversed with sugammadex. He was extubated in the operating room and transferred to the post anesthesia care unit.

DISCUSSION

Desbuquois Syndrome is a rare syndrome with autosomal recessive osteochondral dysplasia [4]. Although it is mentioned that DS has a mortality rate of 33%, the exact mortality can't be calculated when it is considered that there are also milder forms that are not diagnosed [5]. Our patient was also a mild form of DS. The diagnosis of DS of our patient was made when the c375 G>C homozygous mutation was positive in the 2nd exon of the CANT1 gene.

Although there is a risk of a difficult airway, we could not apply regional anesthesia to the patient instead of general anesthesia because the operation time would be long. The use of videolaryngoscopy as the first choice was a factor that facilitated intubation especially in the presence of expected difficult intubation and Coronavirus pandemic precautions. Although inhalation anesthesia was mentioned in the previous case reports, we thought that total intravenous anesthesia was safer in terms of malignant hyperthermia due to musculoskeletal anomalies in DS. We preferred to use total intravenous anesthesia in our case. Total intravenous anesthesia can be used in these patients to avoid the risk of malignant hyperthermia, although it is not clearly mentioned in the literature. Some other anesthetic implications that may accompany DS like joint dislocations (including cervical vertebrae), gastroesophageal reflux, and respiratory and

cardiac involvement should be considered as well as airway management [6,7]. Because of the risk of joint deformity, we performed laryngoscopy gently and carefully. We think that our case will contribute to the literature in terms of being an airway management of an adult case. Adult patients with DS can be managed safely with adequate difficult airway preparation.

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The data of the study were presented for the first time as a poster presentation at the Balkan States Anesthesia Days - VII held on 30 April- 2 May 2021.

Author contribution

Study conception and design: MT, BB, and FS; data collection: HK and MT; analysis and interpretation of results: MT; draft manuscript preparation: AAY and MT. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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CASE REPORT

Persistent Extensive Microcalcifications After Neoadjuvant Chemotherapy: Benign or Malignant?

Gamze Durhan[1]
ORCID: 0000-0002-6281-9287

[1]Hacettepe University, Faculty of Medicine, Department of Radiology, Ankara, Turkey.

Corresponding Author: Gamze Durhan
Hacettepe University, Faculty of Medicine, Department of Radiology, Ankara, Turkey.
E-mail: gamzedurhan@gmail.com

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ABSTRACT

Neoadjuvant chemotherapy is increasingly used for breast cancer due to its several benefits. Assessment of the response to neoadjuvant chemotherapy plays a key role in the management of the disease. Although magnetic resonance imaging is the most accurate method, evaluation of the response to neoadjuvant chemotherapy may be challenging especially in the presence of residual microcalcifications. The presence of residual microcalcifications may not always suggest the residual viable tumor.

In this case, a 48-year-old patient with breast cancer who had persistent extensive microcalcifications after neoadjuvant chemotherapy is presented. Magnetic resonance imaging demonstrated a complete response with the absence of any residual enhancement. Final histopathological results after breast-conserving surgery revealed pathological complete response which is consistent with magnetic resonance imaging and inconsistent with mammography findings. Mammography images showed residual malignant-type microcalcification after surgery, although most of them were excised. However, microcalcifications haven't progressed and recurrent cancer hasn't been observed on MRI and mammography images during the 10-year follow-up.

Keywords: Neoadjuvant chemotherapy, microcalcifications, magnetic resonance imaging, mammography.

INTRODUCTION

Neoadjuvant chemotherapy (NAC) for the treatment of breast cancer has been increasingly used due to several advantages. NAC can decrease tumor size and axillary metastatic nodes before surgery and allow breast-conserving surgery (BCS) and omission of full axillary lymph node dissection (ALND). Furthermore, NAC can provide direct observation of treatment efficacy [1].

Pre- and post-treatment imaging methods including ultrasonography (US), mammography, and magnetic resonance imaging (MRI) are used to assess response to NAC and play a key role in the management of the disease. MRI is superior to other imaging modalities in the evaluation of residual disease after NAC [1,2]. The overall diagnostic accuracy of MRI in predicting pathological response is reported to be 84% [3]. On the other hand, evaluation of malignant microcalcifications after NAC is challenging as they are not observed on MRI, and the presence of residual microcalcifications doesn't always show the viable tumor. So, the decision on surgical approach may become controversial.

Here, a patient with breast cancer who had persistent extensive microcalcifications after NAC is presented. Histopathological results after BCS revealed pathological complete response (pCR) which is consistent with MRI and inconsistent with

mammography findings. While mammography images show residual malignant-type microcalcification after surgery, microcalcifications haven't progressed and recurrent cancer hasn't been observed on MRI and mammography images during the 10-year follow-up.

CASE PRESENTATION

A 48-year-old female patient was admitted to the hospital with complaints of palpable mass within her left breast. Mammography images demonstrated extensive pleomorphic malignant-type microcalcifications and skin thickening in the left breast. Masses could not be observed on mammography secondary to dense fibroglandular tissue (Fig 1a and 1b.) US revealed hypoechoic irregular masses with echogenic foci corresponding to microcalcifications. Contrast-enhanced breast MRI detected non-mass enhancement with type 3 dynamic curve and extensive distribution involving nearly all of the right upper and lower outer quadrants of the breast (Fig 1c). Enlarged lymph nodes without fatty hilum were detected on the right axilla. US-guided core biopsy was performed and pathology results showed both grade 3 invasive ductal carcinoma and ductal carcinoma in situ (DCIS) with comedo necrosis. The tumor was estrogen receptor (ER) positive (50%) with negative progesterone receptor (PR) and positive human epidermal growth factor receptor 2 (HER 2) expression.

The patient underwent four cycles of NAC with cyclophosphamide and adriamycin. Subsequent paclitaxel and trastuzumab treatment was performed for 12 weeks. After NAC treatment, follow-up mammography images showed persistent extensive microcalcifications while MRI demonstrated complete response with the absence of any residual enhancement (Fig 2a, 2b, and 2c). Despite extensive microcalcifications in the right breast, the patient underwent BCS

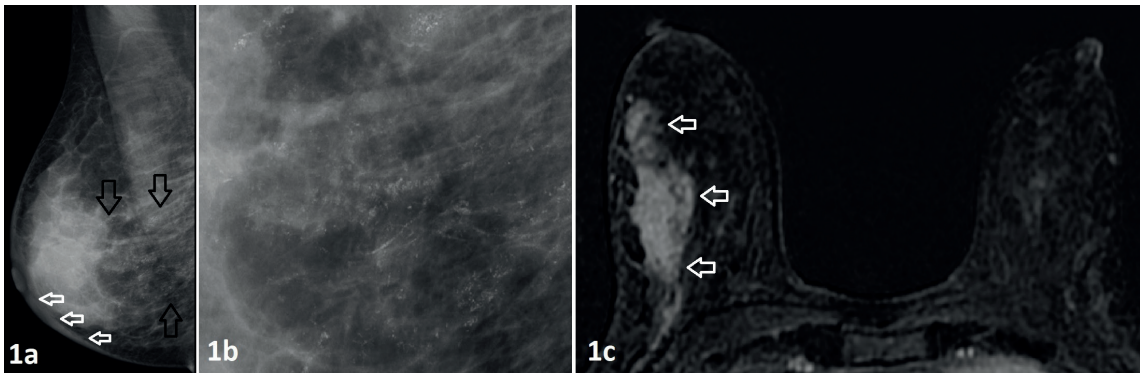

Figure 1. Radiological images of the patient before neoadjuvant chemotherapy.

Mediolateral oblique (MLO) mammography (1a) shows skin thickening (white arrows) and widespread malignant pleomorphic microcalcifications (black arrows). Zoomed image of mammography (1b) demonstrates microcalcifications better. MRI (1c) depicts contrast enhancement in the outer half of the breast (white arrows).

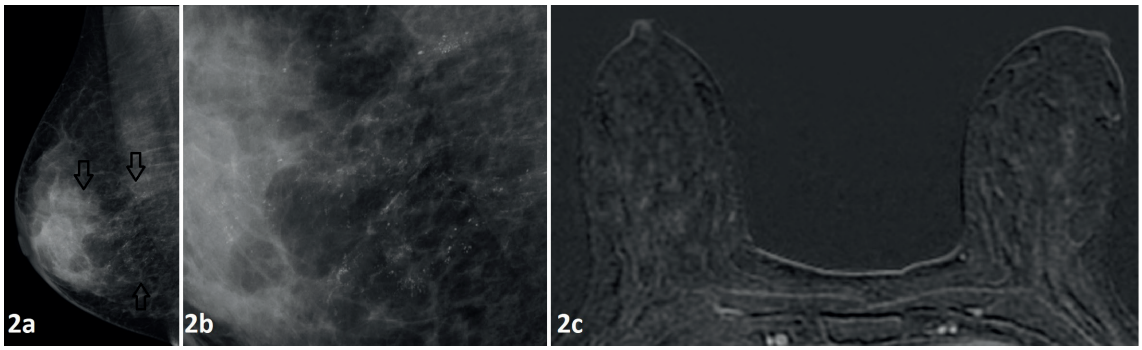

Figure 2. Radiological images of the patient after neoadjuvant chemotherapy.

Mediolateral oblique (MLO) mammography (2a) demonstrates extensive residual pleomorphic microcalcifications (black arrows). Zoomed image of mammography (2b) shows residual microcalcifications better. Complete response with the absence of any residual enhancement is observed on MRI (2c).

upon her preference. Histopathological results of BCS and axillary lymph node dissection revealed the complete disappearance of the invasive and in situ cancer. After surgery, residual malignant-type microcalcifications were observed on mammography images, although most of them were excised (Fig 3a and 3c). But, it was decided to follow up the microcalcifications with imaging methods because of the complete response in the pathology results. During a 10-year follow-up, mammography images did not depict increased microcalcifications and suspicious enhancement was not observed on MRI (Fig 3b and 3d). The follow-up of the patient is still ongoing.

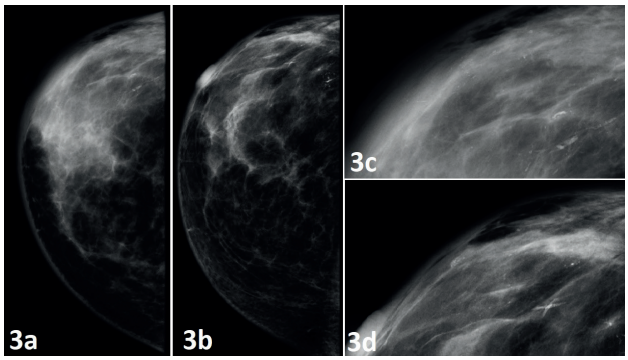

Figure 3. Post-surgical and follow-up mammography images of the patient.

Craniocaudal (CC) mammography (3a) and zoomed mammography (3c) images after surgery demonstrate decreased residual microcalcifications. Residual microcalcifications are still seen on the 10th year follow-up CC mammography image (3b) and zoomed mammography image (3d).

DISCUSSION

In the present case, a patient with breast cancer had persistent extensive microcalcifications on mammography images and did not show any enhancement on MRI after NAC. Histopathological results were consistent with MRI results and showed pCR. Also, residual malignant-type microcalcifications after BCS have not shown malignant transformation during the 10-year follow-up period.

NAC has usually been used for locally advanced breast cancer. NAC can downstage breast cancer and allow BCS, rather than mastectomy. NAC can also avoid the morbidities of ALND such as lymphedema and limb disorders as it increases the usage of sentinel lymph node dissection rather than performing ALND for all node patients before NAC [4]. On the other hand, NAC is increasingly used to evaluate individual tumor response in earlier stage breast cancer. So, NAC can give prognostic information of disease [5]. Although physical examination can be used for assessing tumor response to NAC, its accuracy is inferior to imaging methods [3].

The most accurate imaging method for the evaluation of tumor response to NAC is MRI. The ability to predict the presence of the disease has been found high(93%) while the ability to predict the absence of disease has been detected only mild (65%) according to the final pathology results in a recent study [3]. MRI can underestimate the presence of disease in patients treated with antiangiogenic drugs because of decreasing contrast enhancement [1]. So, complete resolution of contrast enhancement on MRI does not always show the absence of tumor contrary to our case which has a complete response on MRI and at final pathology results. To predict pCR is more difficult when there are accompanying residual microcalcifications on mammography images.

Microcalcifications may decrease or increase after NAC without exact correlation with the presence or absence of residual viable tumor. Residual or newly developed microcalcifications may be the result of necrotic tumor cells, hematoma, fat necrosis, or the development of DCIS [6, 7]. Although residual microcalcifications usually correspond to benign disease such as dystrophic and psammomatous types in histopathological results, complete excision of suspicious microcalcifications are recommended [6, 8]. However, BCS may not be performed in the presence of extensive microcalcification and mastectomy may be needed despite the unwillingness of the patient. BCS was performed due to the preference of the patient in our case. And our patient did not undergo mastectomy with the pCR results despite residual microcalcifications. Regular mammography images did not show increased microcalcifications for 10-year- follow-up and any suspicious enhancement was not observed on MRI performed 10 years after the surgery.

In conclusion, persistent malignant-type microcalcifications may not always be indicative of residual disease. MRI is the most accurate imaging method for the evaluation of

response to NAC. Although the excision of the residual microcalcifications after NAC is usually recommended, patients who have no enhancement on MRI and show pCR at final lumpectomy results may be followed-up carefully.

Author contribution

Study conception and design: GD; data collection: GD; analysis and interpretation of results: GD; draft manuscript preparation: GD. The author reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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LETTER TO THE EDITOR

A Case of Pneumatosis Intestinalis Associated with Sjogren's Syndrome

Seiichi Horinaka[1]
ORCID: 0000-0003-0476-1504

Masashi Ohe[1]
ORCID: 0000-0002-6684-6688

Haruki Shida[1]
ORCID: 0000-0001-6331-0952

Ken Furuya[1]
ORCID: 0000-0003-3373-4337

Misturu Sugiura[2]
ORCID: 0000-0001-6972-7070

[1]Department of Internal Medicine, JCHO Hokkaido Hospital, Sapporo, Japan

[2]Department of Diagnostic Radiology, JCHO Hokkaido Hospital, Sapporo, Japan

Corresponding Author: Masashi Ohe
Department of Internal Medicine, JCHO Hokkaido Hospital, Sapporo, Japan.
E-mail: oektsp1218@sweet.ocn.ne.jp

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A 68-year-old female patient with primary Sjogren's syndrome (SjS) was referred to our hospital due to dry cough exacerbation and abdominal fullness. However, she complained of neither abdominal pain nor constipation. She was diagnosed with SjS accompanied by interstitial pneumonia based on sicca symptoms, positive Schirmer's test, elevated levels of anti-SSA antibody, positive sialography, and honeycomb opacities on chest computed tomography (CT), 7 years ago, and was successfully treated with corticosteroids (CSs), such as prednisolone (PSL) and methyl PSL. During the last 4 years, the patient received methyl PSL at 4 mg/day. On this visit, the clinical presentation showed a distended abdomen and weak bowel sounds, without rebound tenderness. Laboratory findings were as follows: white blood cell (WBC) count of 5,610/μL (of which neutrophil cells were 68.7%); hemoglobin (Hb) level of 11.5 g/dL; platelet (Plt) count of 25.3 × 10^4/μL; C-reactive protein level (CRP) of 0.20 mg/dL; and lactate dehydrogenase (LDH) level of 170 U/L (Table 1). Chest CT scan revealed honeycomb opacities in both lower lung fields, and intra-abdominal free air indicating pneumoperitoneum (red arrow) and dilated intestinal canals, wherein the walls contain air-filled cysts (Figure 1A). Abdominal CT scan also revealed pneumoperitoneum, and dilated intestinal canals, wherein the walls contain air-filled cysts (Figure 1B, C). Based on these findings, the patient was diagnosed as having a complication of pneumatosis intestinalis (PI) and not gastrointestinal perforation. The patient was advised to eat very slowly and prohibit the habit of drinking carbonated water. She was treated with dextromethorphan hydrobromide hydrate for deteriorating dry cough, mosapride citrate hydrate for intestinal hypomotility, and dimethicone for abdominal fullness. However, the aforementioned symptoms did not improve within 3 months after treatments. Furthermore, the abdominal CT findings were exacerbated (Figure 2A, B). Therefore, taking notice of constipation, she received an add-on dihydrocodeine phosphate treatment. Three months thereafter, the aforementioned symptoms and abdominal CT findings considerably improved (Figure 3A, B).

In the present case, PI with pneumoperitoneum mimicked gastrointestinal perforation; however, there was no clear evidence of gastrointestinal perforation and peritonitis because the patient had no abdominal pain and rebound tenderness, and there was no evidence of peritoneal inflammation in laboratory data.

Table 1. Laboratory data

WBC	5,610/μL	TP	6.8 g/dL
Baso μ	0.4 %	Alb	3.7 g/dL
Eosino	1.8%	Na	142 mEq/L
Neutro	68.4%	Cl	108 mEq/L
Lympho	20.7%	K	4.2 mEq/L
Mono	8.4%	Ca	9.1 mg/dL
RBC	383x104 /μL	BUN	11.2 mg/dL
Hb	11.5 mg/dL	Cr	0.38 mg/dL
Plt	25.3x104 /μL	UA	4.0 mg/dL
		Fe	79 mg/dL
TB	0.5 U/L	TG	174 mg/dL
CPK	37 U/L	LDL-C	84 mg/dL
LDH	170 U/L		
ALP	81 U/L	CRP	0.2 mg/dL
GOT	21 U/L		
GTP	13 U/L	FBS	85 mg/dL
γGTP	21 U/L	HbA1c	5.5%

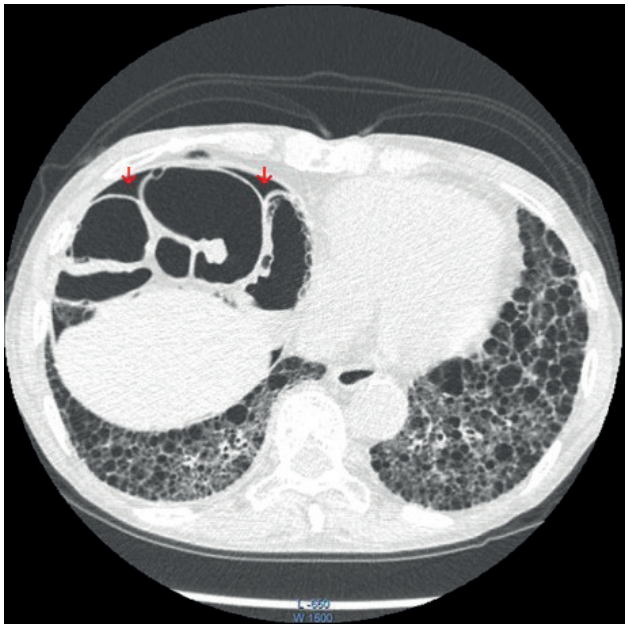
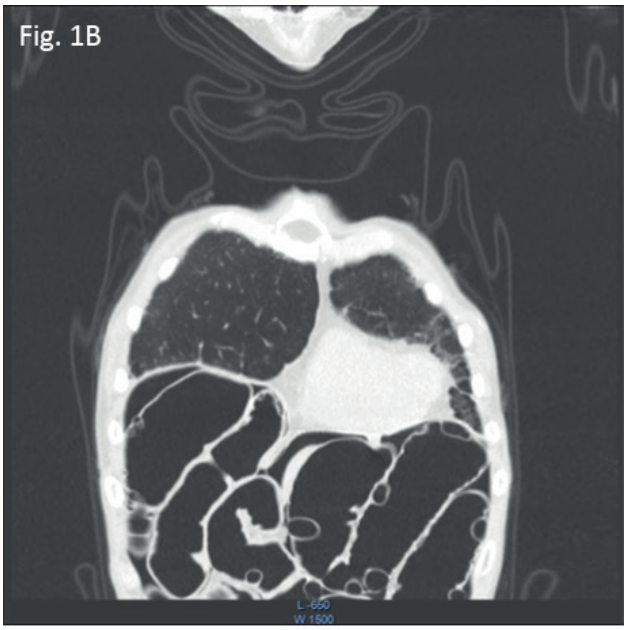


Figure 1A. Chest CT scan reveals honeycomb opacities in both lower lung fields, intra-abdominal free air indicating pneumoperitoneum (red arrow), and dilated intestinal canals, wherein the walls contain air-filled cysts.

PI is a rare disease characterized by the presence of gas in the gastrointestinal wall and is known to be associated with several clinical conditions, such as pulmonary diseases (e.g., asthma, cystic fibrosis and COPD), gastrointestinal diseases, and traumatic injury, as well as connective tissue diseases [1,2].



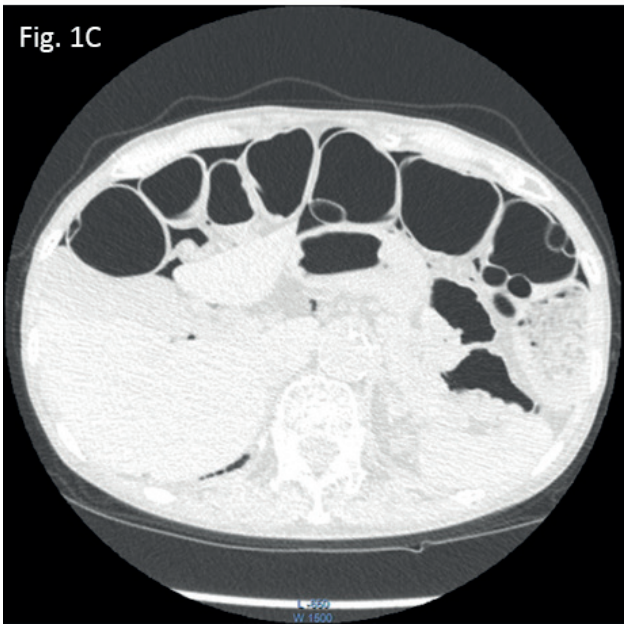


Figure 1B, C. Abdominal CT scan reveals pneumoperitoneum and dilated intestinal canals, wherein the walls contain air-filled cysts.

Especially, PI is commonly seen in systemic sclerosis (SSc) but rarely in systemic lupus erythematosus (SLE) [3]. Similarly, few cases of PI associated with SjS have been reported [3-6]. In patients with SSc, smooth muscle cell atrophy and fibrosis in the gastrointestinal walls are observed, which lead to intestinal hypomotility. Bacterial overgrowth and bowel distention are observed in this condition, which can lead to elevated intraluminal pressure and force gas into the intestinal walls [1]. On the other hand, intestinal vasculitis is speculated to be associated with PI in patients with SLE [7]. Contrarily, etiologies of PI associated with SjS remain

unclear [3]. CSs are reported to be associated with PI. CSs decrease lymphatic tissue in the intestinal Peyer's patch cells, resulting in degeneration of the mucosa that leads to the entry of gas into the peritoneum and intestinal wall [3]. As mentioned above, pulmonary disease is a cause of PI. A severe cough can trigger alveolar rupture, which can result in the introduction of air along the vascular channels in the mediastinum, tracking down to the retroperitoneum, and then to the mesentery of the bowel [1]. Consequently, severe cough is thought to be associated with PI.

In the present case, not only the long-term administration of CSs but also exacerbated cough due to interstitial pneumonia associated with SjS were thought to be one of the etiologies of PI since dihydrocodeine phosphate improved PI. Previous reports regarding PI associated with SjS revealed

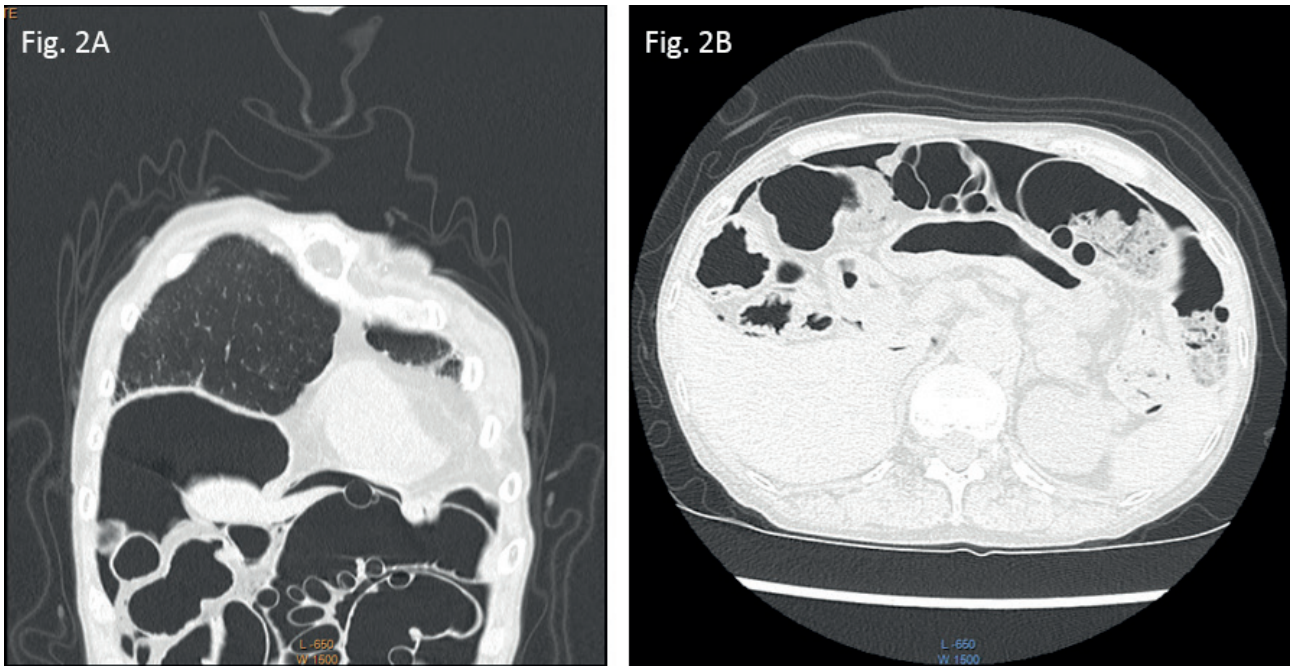


Figure 2A, B. Abdominal CT scan reveals the exacerbation of pneumoperitoneum, dilated intestinal canals, and air-filled cysts of the walls.

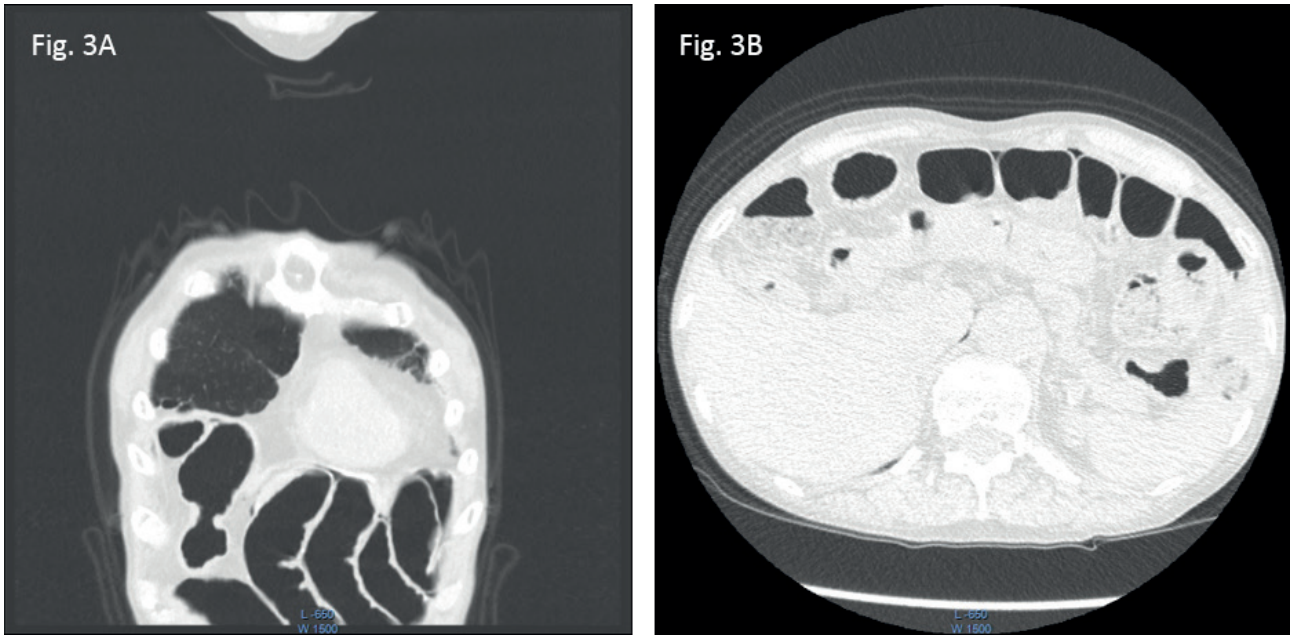


Figure 3A, B. Abdominal CT scan reveals the improvement of pneumoperitoneum, dilated intestinal canals, and air-filled cysts of the walls.

that treatments with cisapride, diet, and oxygen inhalation were efficacious [5,6]. PI associated with SjS is a rare complication, and the etiologies remain unclear. Therefore, more research is required to elucidate the etiologies, as well as prompt and precise diagnosis and efficacious treatments.

Conflict of interest

The authors declare that there is no conflict of interest.

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IN THE HOSPITAL

COVID-19 Pandemic Experience in a Tertiary Care Center in Turkey: What have We Learned?

Yasemin Özsürekci[1]
ORCID: 0000-0001-8662-6909

Özlem Tekşam[2]
ORCID: 0000-0003-1856-0500

Ümit Murat Şahiner[3]
ORCID: 0000-0003-0088-913X

Elif Nursel Özmert[4]
ORCID: 0000-0002-4911-9200

Mehmet Ceyhan[1]
ORCID: 0000-0002-2318-3394

[1]Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Infectious Diseases, Ankara, Turkey.

[2]Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Emergency Medicine, Ankara, Turkey.

[3]Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Allergy and Immunology, Ankara, Turkey.

[4]Hacettepe University Faculty of Medicine, Department of Pediatrics, Ankara, Turkey.

Corresponding Author: Özlem Tekşam
Hacettepe University Faculty of Medicine, Department of Pediatrics, Division of Pediatric Emergency Medicine, Ankara, Turkey.
E-mail: ozlemt@hacettepe.edu.tr

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ABSTRACT

Coronavirus disease 2019 (COVID-19) has become a public health threat to people all over the world and it has stretched hospital resources since the beginning of the pandemic. Available reports to date showed that COVID-19 seems to be less common in children. However, the number of pediatric patients is increasing and a lower number of pediatric patients does not necessarily mean that children are less susceptible to the infection. On the other hand, it is shown that preparedness and response to the COVID-19 disease pandemic in the hospital caring for children are extremely variable. The main target during a pandemic is to maintain high quality and high-efficiency care, with emphasis on patient and provider safety. A documented pandemic plan, simulation training, appropriate use of personal protective equipment (PPE), and appropriate isolation areas in the hospital and also in the emergency department are essential components of pandemic response. Therefore, respiratory hygiene, proper patient placement/ isolation, handling and cleaning of patient care equipment, devices, and environment and procedure safety are all important for effective working flow and reliable working environment in the hospital.

Early recognition and isolation of a patient with COVID-19 may help decrease exposure to the other patients and healthcare personnel. The use of a strict surveillance and management protocol during outbreaks of highly virulent viruses such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), through dedicated patient pathways, adoption of personal protective equipment for health care personnel, and use of nasopharyngeal screening of all admitted children and possibly all healthcare personnel should be recommended. Therefore, we developed a protocol addressing reception, risk management, and hospitalization of suspected SARS-CoV-2 cases at the pediatric emergency department, pediatric wards, and outpatient clinics aimed at containing intrahospital transmission of the infection. Our pandemic response planning was characterized by close collaboration among the head of our hospital, department of pediatrics, pediatric emergency department, pediatric infection control committee, and front-line staff as well as optimization of communication channels. In this article, we aimed to share our experiences of how to handle pediatric patients with COVID-19 in our university hospital from all aspects including prevention of possible transmissions during the first few months of the COVID-19 pandemic.

Key words: COVID-19, severe acute respiratory syndrome coronavirus 2, children, management in hospital, pandemic

INTRODUCTION

An outbreak of novel coronavirus-2019 (COVID-19) infection is a public health emergency of international concern and was first discovered in Wuhan, China [1,2]. The number of cases rapidly increased, particularly in North and South America in addition to Europe which was the most affected region outside of China. At the beginning of the COVID-19 pandemic, pediatric patients were extremely rare [3], whereas, in the later stages of the pandemic, patients with COVID-19 from the whole age spectrum from all over the world [4,5] and in Turkey were reported. In Turkey, the first case of infection with SARS-CoV2 was announced on March 11, 2020. Currently, the documented cases increased up to 8,178,901 on November 20, 2021[6]. Available data regarding SARS CoV2 infections in children on children are limited worldwide as well and confirmed pediatric cases with COVID-19 who had commonly milder disease course compared with adults consisting of 1-5% of all cases with the lack of universal diagnostic and treatment modalities [7,8]. There are still concerns about the transmission route of the SARS-CoV-2. However, respiratory secretions in droplets produced by an infected person during coughing or sneezing seem the predominant way. Additionally, the possibility of airborne spread of COVID-19 and the existence of viable viruses on environmental surfaces and in feces should be taken into consideration [3,9-12].

Hacettepe University Ihsan Dogramaci Children's Hospital is a 272-bed tertiary care pediatric hospital in Ankara, Turkey. It is a pediatric hospital with 16-bed pediatric intensive care unit, 22-bed neonatal intensive care unit, and 4-bed hematopoietic bone marrow unit in addition to general pediatric wards. A total of 273 physicians and/or researchers, 224 nurses, and 429 administrative staff work in the hospital. As of July 14, 2020, 117 confirmed and 235 suspected COVID-19 pediatric cases were admitted to the hospital. Up to July 14, 2020, 6 HCWs were diagnosed with COVID-19, which was attributable to out-of-hospital exposure (the sources were mainly their spouses).

Few data are available regarding management strategies and infection control practices in children with COVID-19 [13,15]. In the period 16 March-14 July 2020, 6 (0.6%) of health care workers (HCWs) were found positive in Hacettepe University Ihsan Dogramaci Children's Hospital and the percentage of positive test results was really low when compared with some hospitals located in Europe [16,17]. We aimed to share our experiences regarding how to handle pediatric patients with COVID-19 in a pediatric referral and tertiary care university hospital from all aspects including prevention of possible transmissions during the first few weeks of the COVID-19 pandemic.

The Algorithm of Pandemic in a Tertiary Care Pediatric University Hospital

Preparation of Hospital and Pediatric Emergency Department (PED)

1. General applications in Hospital

After the declaration of the COVID-19 pandemic worldwide and in Turkey, Hacettepe University Ihsan Dogramaci Children's Hospital implemented a "Pandemic Action Plan" on March 16, 2020 (Table 1) as well as additional measures according to Covid 19 Guidelines created by The Turkish Ministry of Health. Interns (6th-grade medical students) and grade 4 and 5 medical students who have been a part of the trainee program in the hospital withdrew from the hospital environment. All meetings, conferences, and lectures of medical students were cancelled and later completed online. New elective admissions were cancelled and only emergency cases and chronic patients were served. Pediatric residents started to work in shifts to prevent them from contacting high viral load as well as suffering from burnout syndrome.

2. Applications in the Pediatric Emergency Department

Our Pediatric Emergency Department (PED) is the frontline of our children's hospital, serving as the main point for triaging patients as non-infected versus infected (suspected/confirmed patients) with COVID-19 as in many hospital emergency departments.

At the beginning of the pandemic, our major aim was to protect the HCWs from SARS-CoV-2 infections and to prevent the spread of this infection from patient to patient. Therefore, respiratory hygiene, proper patient placement/ isolation, handling and cleaning of patient care equipment, devices, and environment and procedure safety

Table 1. Timeline of key pediatric infectious disease team interventions.

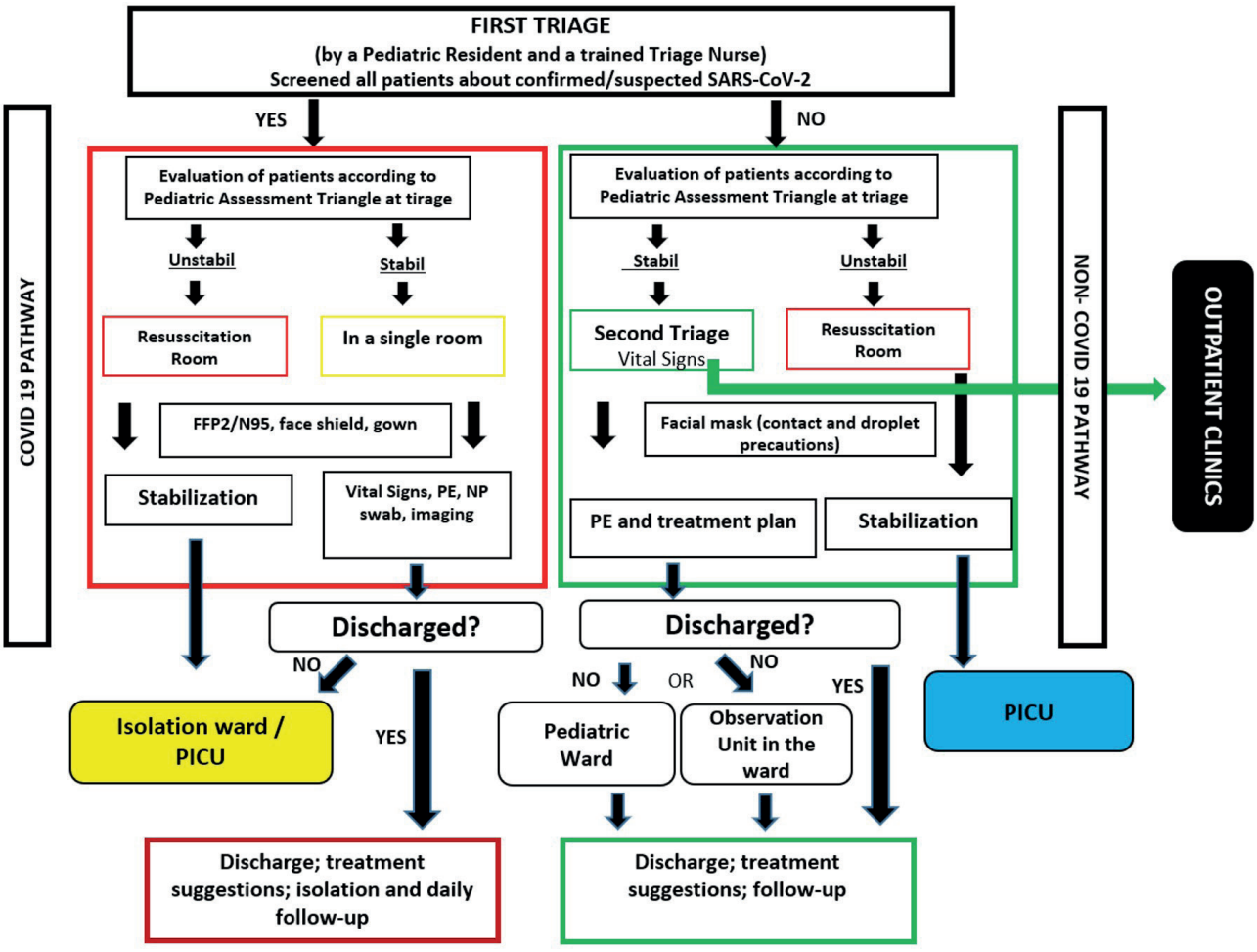
Date	Intervention	Rationale
15-31 Jan 2020	Small groups in hospital, particularly including HCWs of pediatric emergency department, pediatric intensive care unit department, department of infectious diseases etc. started to be notified about disease as well as precautions of hospital infection control committee HCWs were continued to be informed about the last situation of COVID-19 worldwide and updated the infection control practices.	Increasing number of imported cases worldwide
4 Feb 2020	Stock levels of PPEs were strictly started to be monitored via a software program day by day and the certain levels were determined for each PPE in each area of hospital to manage the decline of equipment under the critical threshold.	Possible PPE requirement due to the number of patients likely to increase
10 Feb 2020-11 March 2020	The last meeting in face to face about the last position of COVID-19 pandemic in a big conference hall was organized and HCWs in small groups were continued to be informed about the last situation of COVID-19 worldwide.	Increasing local transmission in several countries.
16 March 2020	Hospital "Pandemic Action Plan" was put into practice	Containment and isolation purposes since the first case in our country were diagnosed on March 11, 2020
18 March 2020	A specific ward consisting of 12-rooms beds with negative airway pressure was arranged and the pressure of rooms were continuously controlled by technical team of the hospital.	Local transmission was reported in Turkey. Reported asymptomatic pediatric cases who had a possibility of high viral load.
19 March 2020	"Diagnosis and Treatment Algorithm for Children" was prepared for our hospital in the light of the accumulated literature and then, was revised two times according to the dynamic nature of pandemic.	Limited data on management procedures of children worldwide.
20 March 2020	Video-assisted training was programmed by Children's Hospital Infection Control committee and shared with target groups on WhatsApp. • Small videos (approximately 5 minutes) about putting on PPE and removing PPE were prepared and shared with all HCWs, including doctors, nurses, and administrative staff who contact with patients and required appropriate use of PPE during pandemic. • Accumulated literature knowledge about COVID-19 from many different aspects was prepared using Zoom: video conferencing and shared via WhatsApp.	Need of continuous education
22 March 2020	"Risk Assessment Score for HCWs" was prepared for the early management of risky behavior of HCWs.	Evidence of infected HCWs with COVID-19 in Turkey
23-26 March 2020	The brochures about "putting on and removing PPE", "management algorithms of pediatric cases", and "warning about confirmed case with COVID-19" were prepared. The rules and brochures about "prolonged or extended use of N95" were prepared.	Increasing local transmission in Turkey. The first pediatric case with COVID-19 was confirmed in our hospital.
1-7 April 2020	Video-assisted education was continued to be programmed by Children's Hospital Infection Control committee • Short new videos (approximately 2 minutes) about putting on PPE and removing PPE were prepared one more time and shared with all HCWs. • Short videos (13 pieces about 2-3 minutes) about cleaning and disinfection procedures were prepared and shared via organized WhatsApp group with the target team. • A literature review on the experiences of different countries about COVID-19 was prepared using Zoom: video conferencing and shared via WhatsApp with the target groups.	Growing number of cases in Turkey
1 June 2020	Beginning of the normalization in the hospital as well as in the country	Decreasing number of cases with COVID-19, although cases increased again.

HCWs: Health care workers, PPE: Personel protective equipment

were all important for effective workflow and a reliable working environment in our PED. Thus, PED in our hospital had five different roles during the outbreak and these roles will continue even though the pandemic is over. These roles can be listed as follow: First, identifying patients with suspected COVID-19 in the pre-triage area (triage 1) by screening and later administering the test for suspected COVID-19 patients. Second, admitting suspected/confirmed patients with COVID-19 to the isolation ward and starting their initial treatment. Third, collaborating and communicating with the head of our hospital, the pediatric infection control team, and the department of pediatrics to initiate the pandemic plan. Fourth, reporting suspected/confirmed cases to the National Health Center/Public Health Department of Ankara. Lastly, to continue to the standard pediatric emergency care like trauma, suicidal attempt, home accidents, surgical emergencies, poisoning, and more.

2a. Triage, Screening, and Initial Approach to Patients at risk for COVID-19

In the PED, our team focused on identifying and isolating patients at risk for COVID-19 infection. Triage was the first step of early recognition and rapid initiation of infection control precautions. Therefore, a clinical pathway amongst pediatric emergency physicians, infectious disease specialists, and Hospital Administrators was created and regularly updated to safely evaluate COVID-19 patients according to our National Ministry of Health COVID-19 guideline during the pandemic (Figure 1). Two triage areas were created in front of the PED: Pre-triage (triage 1), located at the entrance of the hospital, and the non-COVID-19 area called Triage 2, located inside of the hospital. Triage in both areas was applied not only for the PED but for every patient admitted to our hospital. However, almost all admissions were accepted to the PED at the beginning of the pandemic.



PE: Physical examination, NP: Nasopharyngeal swab, PICU: Pediatric intensive care unit

Figure 1. Clinical pathway at the pediatric emergency department from the triage to the disposition for all patients.

During pre-triage, the screening chart included questions about The presence of fever, cough, dyspnea, or signs of a respiratory illness, nausea, vomiting, diarrhea, and contact history with a confirmed/suspected case according to the Turkish Ministry of Health COVID-19 Guideline was asked to all patients and their caregivers/parents. All changes were closely followed and our PEDs and hospitals' working plan were updated according to the principles and criterias defined by the revised guideline of the Scientific Board of the Turkish Ministry of Health. During the screening for COVID-19, the initial triage and assessment were done at least 6 ft or 2 meters away from the patient and started only after the patient and the parents/caregivers (only one) don facemasks. After screening, all of the patients were evaluated according to the pediatric assessment triangle to determine the stable and unstable patients. Unstable and critically ill patients with or without suspected COVID-19 infections were directly taken to the resuscitation room. There was only one resuscitation room in our PED. Therefore, all HCWs in this area wore full personal protective equipment (PPE) including N95/FFP2 or N99/FFP3 with a surgical mask.

If the patient had complaints other than COVID-19 infection or any other diseases and these patients were stable, s/he were directed to Triage area 2. They were screened again by a pediatric resident and/or triage nurse in this triage area.

During the pandemic, not only suspected or confirmed COVID-19 cases but also patients with other complaints including patients which require life-saving interventions, traumatic injuries, poisoning and metabolic problems such as diabetic ketoacidosis continued to be admitted to our PED. In addition, the triage acuity level of presenting patients was higher and the number of less urgent patients admitted to PED decreased markedly.

2b. Pediatric Emergency Department Setting

Pediatric emergency care was given in two different areas for the isolation of the infected and non-infected patients. These two areas are the suspected COVID-19 area and the non-COVID area. The entrance to both of these areas was also separated. When all patients and caregivers/parents entered these areas of the hospital, all patients and their caregivers/parents were asked to wear a facemask to reduce the risk of transmission to others in the immediate vicinity.

Our main emergency area was used as a suspected COVID-19 assessment area. Thus, children presenting with symptoms related to COVID-19 to the PED were separated from other patients. There were no negative pressure rooms in this area, instead, there were single isolated rooms without washrooms for the suspected COVID-19 patients. A radiology imaging room in this area was used for obtaining chest x-rays for the suspected COVID-19 patients. Clinically stable suspected COVID-19 patients identified at the pre-triage were placed in a single room with the door closed. However, critically ill patients and patients contacted with persons contaminated with COVID-19 have been placed in the resuscitation room. After stabilization of the critically ill patients, they have been placed in a negative pressure isolation room with high-efficiency particulate air (HEPA) filtration of the recirculated air in our COVID- 19 isolation service. This COVID- 19 isolation service was located outside of the PED and in a different place from the pediatric ward. After March 27, 2020, a container was placed outside in front of the PED. After this date especially high-risk patients with suspected COVID-19 who had close contact with confirmed COVID-19 cases were evaluated in this container. Portable plain radiography was placed within the container and chest x-ray of the high-risk patients were obtained in it. Alternatively, the radiology imaging room in the main emergency area continued to be used for suspected COVID-19 patients.

Once a possible COVID-19 patient was identified, the appropriate pediatric resident /pediatric emergency fellow and a nurse have been notified in an expeditious manner. The physician took the patient's medical history and performed physical examination. The pediatric emergency nurse obtained the required laboratory investigations of the patients. The most crucial point was to wear a face mask during his/her interactions with health care providers (e.g., performing a physical examination, blood drawn) in the room.

Our pediatric emergency workers used alcohol-based hand sanitizers or washed their hands with soap and water before and after contact with a suspected COVID-19 patient according to the recommendation of the Pediatric Infectious Disease Control Committee. They have been trained for the

appropriate use of PPE according to our hospital guidelines, including techniques to safely doff equipment protecting mucous membranes.

Staff cleaned each room after possible COVID-19 patients left the room according to the recommendations of the Pediatric Infectious Disease Control Committee. This staff followed the droplet, contact, and standard precautions with eye protection. Pediatric Infection Disease Control Committee nurses visited the PED frequently and controlled the compliance of the emergency team to the precautions. Patients leaving their treatment room wore face masks, performed hand hygiene, and were educated in proper respiratory hygiene.

2c. Personal Protective Equipment of Pediatric Emergency Department Health Care Workers

Pediatric emergency care for suspected COVID-19 cases or anyone in the same room or area with such patients was given with standard, contact, and airborne precautions, including the use of eye protection (face shield and/or goggles). If patients were critically ill or if an aerosol-generating procedure (e.g., endotracheal intubation, suctioning of the airway, sputum induction) was needed in other areas, HCWs escalated to airborne precautions with the use of a fitted N95/FFP2 or N99/FFP3 respirator instead of a surgical mask.

All our pediatric residents, pediatric emergency fellows, nurses, and other health care providers practiced wearing and doffing PPE regularly. Doffing of PPE was the procedure with the highest risk of infection during the patient-physician interaction, in terms of the spread of SARS-CoV-2. Therefore, posters adopted by the CDC (Centers for Disease Control) showing a simple step-by-step approach to proper doffing of PPE after evaluation of a suspected or confirmed COVID-19 patient were placed inside the rooms.

2d. Training of Pediatric Emergency Workers and Airway Management

All HCWs exercised caution if aerosol-generating procedures, such as bag valve mask (BVM) ventilation, oropharyngeal suctioning, endotracheal intubation, intubation with video laryngoscopy, nebulizer treatment, continuous positive airway pressure (CPAP), biphasic positive airway pressure (BİPAP), or resuscitation involving emergency intubation or cardiopulmonary resuscitation (CPR) were necessary [18,19]. During this training and in real patients, we used an "aerosol box" which consisted of a transparent plastic cube and two circular ports to perform the airway procedure [20]. However, this box extremely restricted the hand movement of the clinicians, especially during the evaluation of smaller children. Therefore, it was not used frequently.

In patients who had deterioration risk and required PICU (pediatric intensive care unit) care, our health care providers considered noninvasive ventilation (NIV), mechanical ventilation, or extracorporeal life support, if necessary. However, these advanced airway management techniques were performed in our isolation service for COVID-19, if enough time was present. During the preparation period, all of our hospital's storage of NIV devices, high-flow nasal cannula oxygen therapy (HFNC) devices, and ventilators were checked. In an effort to combat nosocomial spread and aerosolization of the SARS-CoV-2 virus, all filters were checked before using NIV and/or ventilator. The presence of a HEPA filter was verified in the expiratory limb of the mechanical ventilator prior to patient use at the beginning of the pandemic. Nebulization therapy for patients with acute bronchiolitis and asthma was not given because aerosol generating procedures increased the risk of infection. Instead of nebulized treatment with metered dose inhaler was given via a reusable holding chamber. In the event of a patient presenting with severe respiratory distress or failed prior use of NIV, intubation was performed by an experienced clinician who was previously trained for invasive ventilation and endotracheal tube intubation. Intubation is a high-risk procedure due to the aerosolization of respiratory droplets. Therefore, endotracheal intubation was performed under airborne precautions, including the use of a fitted N95 respirator and if possible, placement of the patient in an isolation room with negative pressure. The most experienced provider intubated with rapid sequence intubation (RSI). To reduce transmission risk, a surgical mask was placed on the patient over the device, especially a high flow nasal cannula (HFNC) or tracheostomy. Video laryngoscopy was preferred to direct laryngoscopy to increase the distance between the intubator and the patient. To reduce inadvertent contamination by touching one's face or hair, a full head cover including the neck was used. Wrist exposure was minimized with the use of long-sleeved gloves or

vertically taping gloves to the gown. It was observed that circumferentially taping of the gloves made the doffing process of PPE more difficult.

During the pandemic, we had no inadequate access to personal protective equipment.

3. Infection Control Practices for Children with Suspected/Confirmed COVID-19, Organization of Healthcare Facility and Training of Health Care Workers

The main infection control measures taken during the COVID-19 pandemic and our standard practice are shown in Table 1, as well.

3a. General Measures in Pediatric Wards

- During the pandemic, visiting hours were cancelled and the change of parents/caregivers was strictly restricted.
- Mandatory standard precautions including hand-washing practices using water and soap and hand-rubbing via 70% alcohol-based solutions at least 20 seconds applied in all settings for all cases and were reminded repeatedly to HCWs in addition to parents/caregivers.

It was mandatory for HCWs to wear scrubs

- Surgical masks were provided to all HCWs, patients according to age, and parents/caregivers.
- N95/FFP2 or N99/FFP3 respirators were provided to HCWs performing aerosol generating procedures including the sampling of nasopharyngeal or oropharyngeal secretions, intubation, bronchoscopy, and tracheostomy [21].
- HCWs including physicians, nurses, and administrative staff not having contact with suspected/confirmed pediatric cases with COVID-19 were strictly separated from the areas where suspected/confirmed pediatric cases with COVID-19 were evaluated.

3b. Isolation of Suspected/Confirmed Pediatric Cases with COVID-19

A ward had been converted to an "isolation ward" and completely reserved for the monitoring of possible/definitive cases with COVID-19. Each room in this ward had a private toilet and bathroom. We aimed to avoid the contamination of other hospitalized cases. A total of 352 suspected cases were hospitalized in this ward. Patients who were confirmed negative PCR test results for SARS-CoV-2 by July 14, 2020, were transferred to other pediatric wards. When there was a suspicion about COVID-19 in terms of clinical and/or laboratory aspects, the patients were followed up in this ward until discharge. All confirmed cases with asymptomatic and mild disease course were discharged as soon as possible and instructed to quarantine at home for at least 14 days under the notification of The Public Health Department of Ankara to minimize the exposure of HCWs. However, the patients with moderate and critical disease courses were hospitalized in this ward until discharge or death. Moreover, extracorporeal membrane oxygenation (ECMO) and mechanical ventilation via tracheostomy were performed for both cases with critical disease courses in this ward, as well.

3c. Specific measures are taken in the rooms of the isolation ward for suspected/confirmed pediatric cases with COVID-19

Unlike adult hospitals, since the rooms of pediatric hospitals also include parents or caregivers, certain specific measures might be required, particularly when considered a high viral load in the air of the room is considered.

- HCWs wore scrubs that were washed in a cleaning room located in the same isolation ward after usage and they were not allowed to go out with scrubs.
- Disposable food boxes were delivered to the location of HCWs. At the entrance of the rooms, the brochures regarding "putting on and removing PPE" and "prolonged or extended use of N95" were hung on the wall to remind the appropriate use of PPE one more time. At the entrance of the rooms, small tables were placed which included gloves, gowns, masks (surgical and N95/FFP2 or N99/FFP3), bones, overalls, length overshoes, glasses, and face shields. Separate baskets were located outside the room for reusable glasses and face shields to sterilize them with the solution prepared with quarter ammonium after every single use.
- HCWs wore full PPE including N95/FFP2 or N99/FFP3 with surgical masks on before entering the rooms.

- The patients, according to their ages and parents/caregivers were required to wear surgical masks in their rooms during all procedures performed by HCWs.
- In every patient room, patient-specific stethoscopes were placed. Monitors which show the vital signs of patients including blood pressure, oxygen saturation and heart rate were turned in the direction of the door for visibility. During clinical practice, glass doors of rooms were one of the most useful measures used during pandemics to evaluate the vital signs of patients as well as respiratory rate outside the room and to observe the clinical situation of the children, as well.
- Antimicrobial treatments with lower dose numbers or oral antimicrobials were preferred for patients requiring antibiotics to decrease the entrance of HCWs into the rooms.
- Salbutamol inhalation via nebulizer was discontinued and metered dose inhaler with a reusable holding chamber sterilized with the solution prepared with hydrogen peroxide was used instead even if the children were hospitalized in negative pressure isolation rooms.
- After the discharge of a pediatric patient with suspected/confirmed COVID-19, firstly all surfaces including the furniture and frequently touched surfaces in the room were physically cleaned and then disinfected using 1000 ppm bleach solution. The equipment with sensitive surfaces such as monitors, mechanic ventilators, etc. was disinfected using hydrogen peroxide-based solutions. Finally, a device (BioXeco 5) that rotates 360 degrees in the room and sprays 3% hydrogen peroxide was used in patient rooms (Figure 2).

3d. Extending the Use and/or Reusing Respirators

All HCWs who had contact with suspected/confirmed pediatric cases with COVID-19 were required to use appropriate PPE. One of the most important elements of PPE was N95/FFP2 or N99/FFP3 respirators and the appropriate regulation of the use of those respirators for the practice of infection control. Priority for the use of respirators was given to HCWs performing aerosol-generating procedures and working in the isolation ward where pediatric patients with suspected/confirmed COVID-19 were hospitalized [22]. As a result, we had to safely manage the extended use and/or reuse of respirators with proper hand hygiene because of the possibility of supply shortage. We prepared an extended reuse protocol and implemented it into our clinical practice. Therefore, we planned to keep adequate supplies during times of peak demands. Although the extended use and reuse of respirators were reported as 8 hours and 5 uses by CDC [23], respectively per device to ensure a safety margin, both the maximum length of continuous use and the possible number of safe reuses for a respirator were not determined in all settings for all cases. Thus, respirators were used by only a single wearer and commonly reused under the additional training and reminders (e.g., brochures) with the self-physical inspection in our hospital. Respirators were kept in a paper bag or on a paper labeled by the user in a room under UV light (Figure 3) between usages.

4. Clinical Aspects, Laboratory, and Radiologic Imaging

We diagnosed suspected cases, according to our national COVID-19 guidelines. Criteria was changed intermittently by the Coronavirus Scientific Advisory Board in Turkey in response to new data

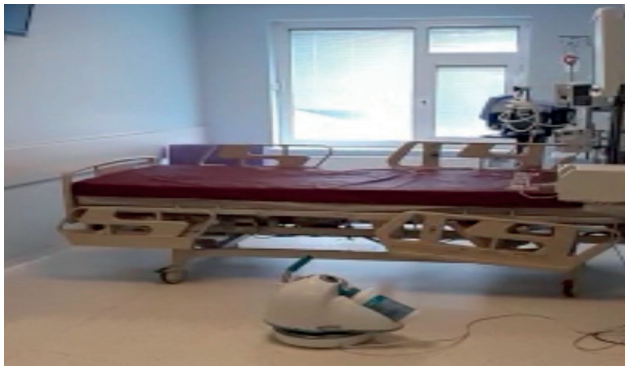


Figure 2. Hydrogen peroxide application in a patient room.

Figure 3. Respirators in a room under UV light between usages.

regarding the disease [24]. Suspected cases with positive reverse transcriptase-polymerase chain reaction (RT-PCR) or serum-specific antibodies against 2019-nCoV were accepted as confirmed cases [13].

A nasopharyngeal swab and bronchoalveolar lavage fluid specimens were collected for RT-PCR testing according to the condition of the patients. However, at the triage, nasopharyngeal swabbing was the preferred sampling method as compared to feces, blood, urine, or others. Moreover, sputum sampling was actually difficult in our case series because of the nature of small children [25]. In dedicated intervals, a nasopharyngeal swab procedure video was shown to all providers [26]. Since the total positive rate of RT-PCR for nasopharyngeal swab samples was reported to be 30-60% at the initial presentation of COVID-19 [27], testing of a specimen of a suspected pediatric case via RT-PCR has been performed a maximum of five times, and positivity was revealed in maximum fourth sampling in our case series. Of all, (18/117) 15.4% of cases were confirmed by testing serum-specific antibodies despite two negative RT-PCR tests. When antibody testing was used after 14 days of the beginning of the symptoms, sensitivity was over 90% [28]. Therefore, RT-PCR from nasopharyngeal swab samples and/or antibody testing of blood samples were used to confirm the diagnosis of COVID-19 in our case series. Additionally, respiratory viral and/or bacterial panels for alternative diagnoses such as influenza or S. pneumonia have been considered for almost all patients under investigation. Complete blood count, CRP, procalcitonin, and chest x-ray were ordered for all patients. However, other investigations such as liver function tests, coagulation parameters, ferritin, and troponin were done according to the patient's clinical findings.

A clinical algorithm was created according to the severity of the pediatric cases; moreover, it was revised as our knowledge and experience accumulated. The severity of cases was categorized based on the clinical characteristics, laboratory results, and imaging findings as described [29]: a) asymptomatic infection; no clinical and/or radiological findings, b) mild disease; acute upper respiratory tract infection without clinical and radiological pneumonia, c) moderate disease; pneumonia with the symptoms of respiratory tract infection, d) severe disease; progressive respiratory disease with dyspnea and central cyanosis, e) critically ill cases; acute respiratory distress syndrome or respiratory failure, shock, organ dysfunction including encephalopathy, myocardial injury, coagulation abnormalities, and acute kidney injury.

Chest computed tomography (CT) seems to be a crucial radiological modality for the diagnosis of adult cases with COVID-19 showing typical imaging features like ground-glass opacities, multifocal patchy consolidation, and/or interstitial changes with a peripheral distribution [30]. However, a great majority of children had mild or moderate disease without severe pulmonary involvement, which was compatible with our results [7,13]. Therefore, radiological assessment of our pediatric cases with a suspicion of COVID-19 was coupled with clinical and laboratory examinations. As a result, CT was not used routinely for diagnosis because of its low diagnostic value and harmful effects on children and was performed only for patients with severe lower respiratory tract symptoms. When we decided whether to use CT, ultrasound (US) was often preferred as an alternative radiological modality. Ultrasound is repeatable and reliable, has no radiation, and is an inexpensive method. We used lung US as a way to screen patients with suspected COVID-19 pneumonia. Lung US is superior to standard chest radiography, with the additional advantage of the ease of use at the point of care, repeatability, absence of radiation exposure, and low cost [31].

Moreover, literature reports demonstrate that chest X-ray has a low sensitivity in the diagnosis of pulmonary involvement of COVID-19 [32]. Studies conducted in adults have shown that US results were correlated with chest CT, and US seemed as a reasonable diagnostic method in adult cases with COVID-19 pneumonia [33,34]. There is also an increasing number of studies about children which compared US and radiography features of pediatric cases with COVID-19 [35]. We preferred to use the US in addition to a chest x-ray as a diagnostic tool. The only problem with our US machine was it was not wireless. After use, it was cleaned with hydrogen peroxide.

Although data associated with the myocardial injury caused by COVID-19 is limited, particularly in the pediatric population, the cardiovascular system seems to be the second target of COVID-19.

Patients with COVID-19 have presented with dysrhythmias, acute myocardial infarction, heart failure, myocardial injury, and even myocarditis from the start of the pandemic [36]. Contrary to popular belief, pediatric COVID-19 may be more serious than expected as in our cases, and serious conditions such as multisystem inflammatory syndrome in children (MIS-C) may arise which is related to the exposure to SARS-CoV-2 [37]. Moreover, myocarditis may have been underestimated because of its subtle symptoms in children and since fatality is commonly based on the lung involvement of the virus, clinicians can mainly focus on the respiratory tract symptoms of the patients [38]. Manifestations of myocardial involvement such as ejection fraction decline and myocardial enzyme elevation in addition to fulminant myocarditis have ranged between 10-20% of the patients with COVID-19. It should also be noted that cardiac involvement of COVID-19 may also occur without accompanying symptoms of respiratory tract infection [38,39]. Troponin elevations in patients with COVID-19 might have been directly associated with an increased risk of fatal outcomes [36]. However, as in our cases, the rapid resolution of systolic dysfunction together with mild troponin increase has suggested that the mechanism of systolic dysfunction is not only attributed to the myocardial damage [37]. Further data may have possibly shown us the actual mechanisms of cardiac involvement. We added a cardiac enzyme profile including troponin as well as electrocardiography routinely in the basic evaluation of pediatric cases in our hospital, particularly after we experienced pediatric cases consistent with the multisystem inflammatory syndrome in children (MIS-C).

5. Treatment procedures and follow-up of the confirmed pediatric patients

Currently, there is no specific treatment for patients with COVID-19. Supportive care is the mainstay of treatment, especially in children. Of approximately 1400 pediatric cases in a systematic review including one of the largest pediatric case numbers, 1.9% were admitted to PICU and required mechanical ventilation (MV). Nebulized interferon (IFN) was the most preferred agent for pediatric cases (51.5%), followed by other antivirals (38.4%) and antimicrobials (23.3%). The authors less frequently described the use of intravenous immunoglobulin (IVIG) and corticosteroids (5.2% and 5.8%, respectively) [8]. Of all patients, (1/117) 0.8% were required HFNC, (4/117) 3.4% MV and two of four cases needed ECMO in our case series. Additionally, corticosteroids were not used in any case and IVIG was used for the cases with severe/critical ill disease course (3/117, 2.5%), and anticoagulant therapy was used only for one case with critical disease course.

There is no clear evidence associated with the safety and efficiency of antiviral treatment of children with COVID-19. The development of antiviral treatment was a dynamic process because of the changing and accumulating data in time. During the first days of the pandemic, we prepared a management algorithm that was mainly based on symptomatic and supportive care of pediatric cases without any antiviral targeted therapy. Because the published clinical treatment experiences mostly consisted of descriptive reports and case series from China and early affected countries from the pandemic. Additionally, CDC [40] and WHO [41] highlighted the absence of available specific treatment for patients with confirmed COVID-19 [42]. Although a number of clinical trials were performed to determine the effectiveness and safety of remdesivir, lopinavir/ritonavir, favipiravir, chloroquine/ hydroxychloroquine, and other drugs, few studies involved pediatric cases [43,44].

Many adult-based studies have postulated the mentioned drugs to be effective, whereas the ineffectiveness of some of them such as hydroxychloroquine was published recently despite some of these studies were retracted from the journals. These conflicting findings and suspicious data, as a result, lead us to evaluate the management procedures of cases with COVID-19, particularly in childhood where there is a huge knowledge gap. Lopinavir/ritonavir and arbidol were used in a couple of pediatric case series from China [45], whereas another expert team from the same country [13] did not recommend the use of lopinavir/ritonavir, ribavirin, or chloroquine phosphate in children in their second consensus statement. In a study from North America, remdesivir, hydroxychloroquine with/ without azithromycin, tocilizumab, and convalescent plasma were the therapeutic agents in children with a severe disease course [14]. Since it was reported in a current randomized, controlled trial

that no beneficial effect of the use of lopinavir/ritonavir was present beyond the standard care in adult cases with COVID-19 [46], we did not prefer to use lopinavir/ritonavir from the beginning of the pandemic. A guideline prepared by The Science Board of Turkish Ministry of Health was published on April 12, 2020 including hydroxychloroquine with/ without azithromycin as a first choice and lopinavir/ritonavir in progressive disease. The revised guideline was published on June 3, 2020, included hydroxychloroquine as the first choice and lopinavir/ritonavir or favipiravir in progressive disease. However, none of the pediatric cases with asymptomatic or mild disease courses were treated with an antiviral agent. The Public Health Department was notified and patients with no significant comorbidities without concern for the deterioration of clinical condition were discharged as soon as possible and were instructed to quarantine at home for 14 days starting from the positive test date. During the first period of the pandemic, we planned to use hydroxychloroquine for the pediatric cases with a disease course that ranged between moderate and critical illness. Therefore, hydroxychloroquine was used in 4 children (4/117, 3.4 %) as a single agent in one pediatric case with a moderate disease course and in combination with favipiravir in the remaining 3 patients with a critical disease course. In the following days, increased radiological abnormalities in chest CT images despite antiviral therapy were detected in two of these patients while their clinical condition was improving. During the second period of the pandemic, none of the antiviral agents, except for one case with a critical disease course (favipiravir was used as a single agent), was used for the treatment of pediatric cases with COVID-19. Later, since it has been proven to be harmful, this recommendation was no longer used.

6. Conclusion and Future Direction

SARS-CoV-2 is a novel coronavirus that has affected an unprecedented number of people in the world. Patients typically presented with a combination of fever or cough and had a history of exposure to a person with COVID-19 or had traveled to an affected geographic area at the beginning of the pandemic. While most patients have mild disease, some may develop severe complications including ARDS and multi-organ failure. No curative treatment is currently approved. Early recognition and isolation of a patient with COVID-19 may help decrease exposure to other patients and healthcare personnel. During the pandemic, handling problems in unity, making clinical decisions together, and acting as a team including the head of our hospital, The Department of Pediatrics, the Pediatric Emergency Department, and the Pediatric Infectious Control Committee were important for success. Hopefully, this team will also stimulate further collaboration during the recovery period. From the beginning of the pandemic, the typical organization and structured workflow of our hospital were largely reorganized when anticipating how to limit the infectious spread and care for all patients. Our main target was to maintain high quality and high-efficiency care, with emphasis on patient and provider safety during the pandemic. We think that the key to success was to perform great teamwork during such kind of disaster. Otherwise, a single problem link in the chain could disrupt the whole process.

Lessons Learned:

1. Accurate and reasonable pre-triage is very important to screen for all patients who need care in PED or outpatient/inpatient clinics to keep the hospital "clean". Furthermore, accurate use of PPE in every setting of the hospital will be beneficial to maintaining "clean" areas.

2. Training of the HCWs again and again for personal protection and continued evaluation of compliance with the isolation rules are the crucial steps to maintain a healthy workplace. This may significantly reduce the contamination rates within HCWs.

3. Almost all our pediatric patients had close contact with confirmed/suspected SARS-CoV-2 cases who were commonly a family member at least within 14 days. Therefore, social isolation even within the family and among relatives is important.

4. SARS-CoV-2 infection seems to affect children less commonly and less severely than adults.

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Author contribution

Study conception and design and draft manuscript preparation: YÖ, ÖT, ÜMŞ, ENÖ, MC. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Abbreviations

COVID-19	2019 novel coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
HCW	Health Care workers
HEPA	High-efficiency particulate air
PED	Pediatric Emergency Department
PPE	Personel protective equipment
BMV	Bag valve mask
CPAP	Continuous positive airway pressure
Bi-PAP	bi-phasic positive airway pressure
CPR	Cardiopulmonary resuscitation
CDC	Centers for Disease Control
PICU	Pediatric intensive care unit
NIV	Noninvasive ventilation
HFNC	High-flow nasal cannula oxygen therapy
RSI	Rapid sequence intubation
ECMO	Extracorporeal membrane oxygenation
CDC	Center for Disease Control
RT-PCR	Reverse transcriptase-polymerase chain reaction
CT	Computed tomography
US	Ultrasound
MIS-C	Multisystem inflammatory syndrome in children
MV	Mechanical ventilation
IFN	Interferon
IVIG	Intravenous immunoglobulin