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## Contents

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REVIEW

# Spleen Derived Immune Cells in Acute Ischemic Brain Injury: A Short Review

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Spleen-derived immune cells are considered to play central role in the progression of ischemic brain damage contributing to both the local and systemic inflammatory response initiated by an ischemic insult in the brain tissue. Brain-spleen communication in acute ischemic brain injury has been studied especially in rodent models of stroke, which mimic the acute focal brain ischemia in humans. Rodent spleens decrease in size after experimentally induced stroke, due mainly by the release of spleen`s immune-cells into the circulation. Splenectomy prior to middle cerebral artery occlusion is protective to the ischemic brain resulting in decreased infarct volume and reduced neuroinflammation. Various therapeutic strategies in clinical use aiming to protect the neural tissue after stroke were found to involve the modulation of splenic activity, altogether indicating that the spleen might be a potential target for therapy in ischemic brain injury. Importantly, the most clinical studies demonstrated that the splenic response in stroke patients is similar to the changes seen in rodent models. Thus, despite the limitations to extrapolate the results of animal experiments to humans, rodent models of stroke represent an important tool for the study and understanding of brain-spleen communication in the pathogenesis of acute brain ischemia.

**Keywords**: experimental stroke model, splenocytes, neuroinflammation

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## Introduction

Stroke, with ischemic stroke accounting for almost 90 % of the cases, is the second leading cause of death in middle-income countries, following ischemic heart disease [1]. Its global public health importance is well reflected by statistical data: in the high-income countries, it is the first cause of long-term disability, significantly increasing health spending worldwide [2].

Acute brain ischemia affects the brain parenchyma generating two different damaged areas: the ischemic core, the central region of the brain area to which blood flow is lost and the penumbra, which retains residual perfusion from collateral blood vessels. The latter covers almost half of the total tissue damage volume during the initial stages of stroke [3]. Rodent models of ischemic stroke using transient or permanent occlusion of the middle cerebral artery (MCAO) mimic the acute focal brain ischemia in humans and reproduce confidently the pathology seen in humans [4].

The penumbra shows a remarkable susceptibility to merge into the ischemic core, therefore represents an important target region for salvage both via post-ischemic and preventive therapy [5]. The consequences of acute brain ischemic injury extend far beyond the brain [6]. A variety of immune cells, not only in the central nervous system (CNS) but also in the periphery is activated soon after a stroke. They play a determinant role in the progression and outcomes after stroke [7]. The ischemic neuronal damage involves various pathways, like anoxic depolarization, perturbed glutamatergic and GABAergic neurotransmission and intracellular calcium signaling as well as excessive formation of reactive oxygen species [8], [9]. This complex process activates the local microglia, regarded as resident immune cells in the central nervous system (CNS). Activated microglia will generate chemotactic signals leading to a significant infiltration of peripheral immune cells into the damaged brain area [10]. The peripheral immune cells migrate through the compromised blood-brain barrier and contribute to the brain damage or repair processes after ischemic stroke [11]. The spleen is the most important immune cell reservoir of the body. High number of experimental studies document that the spleen plays a decisive role in the stroke-induced immune response and neurodegeneration [12,13]. The activation of the sympathetic nervous system following an ischemic insult in the brain results in splenic contraction followed by the mobilization and release of different immune cells from this reservoir contributing to the systemic inflammatory response initiated by the acute brain ischemia [14]. The sequence probably is determined by the nature of the ischemic trigger and by the pattern of secreted cytokines/chemokines [12]. The role of the brain infiltrating immune cells in stroke evolution seems to be a dual one by enhancing neurodegeneration [15], or protecting neurons [16], [17]. The splenic origin of brain infiltrating cells after cerebral ischemia was demonstrated using carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, a method which enables the following of migrating splenocytes after experimentally induced stroke. These studies evidenced that after experimental stroke immune cells exit the spleen, their number reducing here significantly after 24-48 hours, and migrate into the damaged brain tissue contributing to injury [12,13,18]. However, inflammatory cells have been shown to exert also protective effects, and

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contribute to post-stroke recovery [19], altogether underscoring the multiple facets of the inflammatory process in stroke. Study of the inflammatory pathways, with their variety of cells and mediators involved in the early stages of neuroinflammation in cerebral ischemia, is difficult to carry out in humans. The many structural overlaps in the histology of human and rodent spleen histology [20-22], makes the rodent spleen suitable for studying the splenic response to stroke. Indeed, the results of the last ten years support that several features of brain-spleen communication after stroke described in experimental studies can be extrapolated to human subjects [23,24] and are promising for integration of these results into the therapy targeting the immune system after stroke. In this short review we analyzed previous PubMed indexed experimental and clinical studies investigating the role of spleen-brain communication in the pathophysiology of ischemic stroke. We focused on recent publications providing data on changes in the cellular components of the spleen after ischemic stroke and evaluating the role of spleen derived immune cells in the progression of ischemic brain damage.

## Splenic changes after ischemic stroke

The spleen has been proved to be the most pro-inflammatory organ following acute ischemic injury at different sites in the body (brain, liver, intestine, kidney, and heart) [25-28]. The changes in the spleen after stroke include mainly three aspects: spleen morphology, numbers of immune cells derived from the spleen and inflammatory cytokine production by the spleen`s cells [15,29]. The released pro-inflammatory cytokines promote a secondary inflammatory response in the brain contributing to amplification of neural tissue damage [12].

The microscopic examination of the spleen removed after acute cerebral ischemia reveals significant morphological changes compared to the normal spleen. These include depletion of lymphoid tissue with reduced or lack of germinal centers [14], decrease in the number and frequency of apoptotic cell death in splenocytes (4 days after transient ischemic insult MCAO mice show a 90% reduction in splenocyte numbers compared to the sham-operated animals), changes in cellularity and phenotype of lymphoid cells and, in general, splenic atrophy. [30]. The spleen contributes to the systemic inflammatory response and neurodegeneration via peripheral immune cells: splenic leucocytes such as various subsets of T and B cells, Mo/MF, polymorphonuclear neutrophils (PMNs), natural killer cells (NK) and follicular dendritic cells (DCs) [12]. Following stroke, due to the activation of the sympathetic nervous system, production of chemotactic cytokines and antigen presentation by the damaged tissue the splenocytes are released into the circulation and reach the damaged brain tissue [12]. There are still controversies concerning the time course of the recruitment of inflammatory cells into the brain as well as their pathogenic roles in the ischemic brain injury. Studies using CFSE labeling demonstrated that splenocytes appear relatively late in the damaged neural tissue, usually days (48 - 96 h) after ischemic brain injury occurs [18].

## Neutrophils

Several studies indicate the neutrophils as the first peripheral cells that infiltrate the brain after ischemic injury (from 30 minutes to 3 days) [11,31,32]. However, recruitment of other inflammatory cells into the brain prior to neutrophil infiltration in response to cerebral ischemia has also been observed [10]. Neutrophils contribute substantially to many aspects of the brain damage occurring after ischemia by releasing ROS, proteases, cytokines and chemokines as summarized in a recent review by Jickling et al [33]. PMNs expressed matrix metalloproteinase 9 (MMP-9/gelatinase B), a member of the family of zinc-dependent proteases has been linked to the disruption of the blood-brain barrier via degradation of the basement membrane and tight junction proteins, followed by edema formation, neuronal death and erythrocyte extravasation [34,35] in animal experiments. High levels of MMP-9 were found in peripheral blood samples of patients with ischemic stroke [36], and an increased number of MMP-9 positive cells was detected in human post mortem ischemic brain tissue, in association with PMNs and activated microglial cells [37]. Quantification of myeloperoxidase (MPO)-labeled neutrophils after permanent MCAO in the damaged brain tissue of rats demonstrated that infarct size significantly correlates with the number of neutrophils around the infarct, larger infarct being accompanied by more neutrophils. In the brain of rats splenectomized 2 weeks before MCAO the number of neutrophils was significantly decreased without significant changes in blood leukocytes, which might contribute to the observed protective effects of splenectomy after ischemic brain injury [38]. Thus, these findings evidence that neutrophils have a negative effect following cerebral acute ischemia and indicate that their inhibition including MMP-9 inhibition might represent a potential therapeutic intervention in stroke [32,39].

## Lymphocytes

The pathophysiological importance of lymphocyte accumulation and their interaction with PMNs into the damaged brain tissue following stroke is not clearly defined [40]. T lymphocytes are considered central players in the development of a sustained inflammatory response after stroke. The massive reduction of splenic immune cells, especially B and T lymphocytes and the concomitant activation of the sympathetic nervous system are considered the main causes leading to a persistent immunosuppressed status of stroke patients, responsible for the increased susceptibility of these patients to post-stroke infections [14]. Although the number of blood lymphocytes declines early after an ischemic insult, the most studies indicate that they appear relatively late in the brain, usually days after the onset of brain injury. Some studies in rodent models demonstrated

accumulation of T cells in the brain already within the first 24 h after focal cerebral ischemia influencing the evolution of brain injury [41,42]. There is a time shift in the distribution of different T cell subsets, which play differential roles in response to cerebral ischemia. The early appearing T-cell subsets after stroke (day 3 to 7) are represented by helper $CD4^+$ (day 3 to 7) and cytotoxic $CD8^+$ T-cells (10). Experimental studies connect the key role of helper $CD4^+$ Th1 cells in the pathogenesis of stroke to the release of proinflammatory cytokines (e.g. interleukins, such as IL-2, IL-12, IFN-γ, tumor necrosis factor -TNF-α) promoting brain damage, however some cytokines (e.g. IFN-γ) are critical for the prevention of post-stoke infections [43,44]. $CD4^+$ Th2 cells may play a protective role through production of anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13) [45,46]. Neo-antigens originating from the neural cells detritus, such as microtubule-associated protein 2 (MAP 2), NMDA receptor subunit NR-2A, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), released into circulation are captured by antigen-presenting cells, especially DCs and macrophages. It is thought, that these stimuli finally trigger the activation of T-cell-dependent adaptive immune responses in the T-cell zone of the spleen [47]. In parallel to the decrease in the total number of splenic immune cells in the early days after stroke, increased number of CD4+FoxP3+ Tregs has been observed in the ischemic brain. Several studies suggested Tregs to exert beneficial effects on stroke evolution. Depletion of Tregs increased tissue loss and worsened neurological functions, probably due to reduced IL-10 production [12,48,49]. In contrast, other studies reported that Treg depletion did not affect stroke infarct volume or even reduced infarct size and improved neurologic function after MCAO, indicating a detrimental Treg effect after experimental stroke [50]. The summary of preclinical studies though indicates an overall neuroprotective effect of Treg-targeted therapies (adoptive transfer of purified polyclonal Treg) in models of stroke, making it a potential candidate for therapy in a specific group of patients with ischemic stroke [51]. Tregs are known to suppress, modulate the activity of other immune cells, including CD4+ and CD8+ T-cells, B-cells, NK cells and circulatory CD11b+ monocytes [52]. Findings revealed that γδT cells, a small subset of T cells that bear a distinct TCR on their surface might also be involved in the pathogenesis of ischemic stroke, TCR-γδ knockout mice, as well as mice treated with TCR-γδ–specific antibody presenting decreased infarct volume [53]. The complex role of lymphocytes in the immune response to stroke is highlighted also by the study of Chen et al. Using flow-cytometric analysis, this group documented that remote ischemic preconditioning of a limb (reported as a protective method against ischemic stroke) followed by MCAO significantly is associated by reduced brain infiltration of $CD8^+$ T cells and NKT cells, increased splenic volume and elevated lymphocyte number in the spleen, including B lymphocytes [54]. The role of B-lymphocytes, the major component of splenic withe pulp [55] in ischemic brain injury is poorly investigated. The low number of reports on this topic provided discrepant results indicating beneficial or neutral effects of the B lymphocyte infiltrate on ischemic brain injury [56,57]. A recent study using pharmacological B cell depletion, B cell transgenic mice, and adoptive B cell transfer experiments disclosed that B cells did not influence infarct volume and functional behavior in mice after acute ischemic stroke [58]. Moreover, Doyle et al. observed B-lymphocyte infiltration of the injured brain, which could contribute to the intensification of the cognitive deficits after stroke [59]. Performing immunostainings of human postmortem tissue, the same group detected B lymphocytes also in the brain of some patients with stroke supporting a B lymphocyte response to stroke also in humans [59]. In contrast, Bregs secreting IL10 had a protective role in ischemia/reperfusion injury in mice due probably by post-stroke immunosuppression [60].

## NK cells

*NK cells*, an important part of the innate immune system, have cytotoxic properties. Following stroke, these cells migrate from the spleen into the infarcted area of the brain together with T lymphocytes and monocytes [18]. The protective effects of splenectomy before acute brain ischemia probably imply also the reduction of NK cells in the damaged area of the brain [61].

## Monocytes/macrophages (Mo/MF)

The local microglia and peripheral macrophages are among the first responders to cell damage in the CNS and are mobilized to the site of injury within hours [62]. The spleen is the main source of monocyte intake in ischemic injury [13]. The structure of the spleen`s marginal zone (MZ) plays an important role in the distribution of the spleen macrophage population. The MZ outer ring contains resident MZ macrophages (CD209b+) that present processed antigens to MZ B cells. The inner rim of the MZ is lined to CD169+ metallophylic macrophages that transfer captured antigen to DCs for activation of the cytotoxic (CD8+) T cells. Macrophages are also present in the red pulp that are F4/80hi and help maintain blood homeostasis by phagocytosis of senescent erythrocytes and blood-borne particulates and their phenotype differ from that of macrophages associated with MZ [20,22].

The spleen contraction after stroke is accompanied by a decreased number of macrophage subsets in the spleen. The displacement of these macrophage subsets from the spleen was found to temporally coincide with increases of the respective macrophage subsets in the ischemic brain [13]. Research over the past decade has evidenced that spleen-derived mouse monocytes can be divided into two distinct populations, each having a different effect on ischemia outcome: the Ly6Chi / CCR2+ subset is pro-inflammatory and the Ly6Clow / CCR2- subset has anti-inflammatory

effects. The Ly6Chi/CCR2+ monocyte subset is specifically recruited in acute ischemic conditions by the monocyte chemoattractant protein-1 (MCP-1), secreted by the cells of the inflamed tissue, and will become classically activated M1 macrophages, with pro-inflammatory phenotype. The Ly6Clow / CCR2 - subset is recruited to the normal tissue and develops into resident M2 macrophages, which have host defense and repair functions after injury [63]. In a previous study CCR2-null mice were protected against cerebral inflammation following brain ischemia, suggesting the important role of CCR2 in stroke-induced brain injury [64]. The polarization of macrophages in a classic pro-inflammatory (M1) or alternative anti-inflammatory (M2) phenotype thus depend on specific environmental signals that induce these different polarization states and consequently determine the function of microglia and Mo/MF [65]. The factors that drive the activation of microglia/macrophages include cytokines, chemokines, released degradation products, and extravasated molecules [63]. Studies document that toll-like receptors (TLRs) are essential players in the process of macrophage activation [63]. Interestingly, it has been found that TLRs play a role in the inflammatory response to ischemic injury even in the absence of infection. More specifically, stimulation of TLRs through TLR ligands and INF-γ induces classical M1 activation either 1) by inducing NF-ƙB, which in turn upregulates pro-inflammatory cytokines (TNFα, IL-12, suppressor of cytokine signaling-3 (SOCS3)) and hypoxia inducible factor 1a (HIF-1a) to promote inducible nitric oxide synthase (iNOS) synthesis, or 2) by inducing interferon regulatory factor 3 (IRF-3), one of the main transcriptional regulators initiating M1 polarization (via signal transducer and activator of transcription 1 (STAT1)) and M2 gene silencing. Against it, IL-4/IL-13 stimulation favors alternative M2 activation. Recently, the triggering receptor expressed on myeloid cells 2 (TREM2), an anti-inflammatory receptor was suggested as an important player in controlling microglial M1/M2-like phenotypes, its deficiency exacerbating ischemic damage in experimental stroke [66]. Many markers (e.g. CD68, CD200, F4/80, CD14, HLA-DR, TLRs, heat shock protein (Hsp)-70, C3b/iC3b, CR3, sodium-calcium exchanger (NCX)1 antigen) are used to denote the reactive status of the macrophages, but they do not define whether macrophages have toxic or protective functions. The classification of macrophages into M1 or M2 subgroups is once again difficult because of the overlap between the antigenic structures. For example, major histocompatibility complex (MHC) class II (involved in antigen presentation to immune cells) and CD86 (functions as a co-stimulatory signal for T-cell activation) do not clearly belong to the M1 or the M2 phenotype. However, despite the antigenic heterogeneity, generally is accepted, that M1 phenotype markers include CD16, CD32, CD86, and inducible nitric oxide synthase (iNOS), while the M2 phenotype expresses arginase-1 (Arg1), CD163, and CD206 antigens [67]. A growing number of published data based on conventional molecular analysis-immunostaining, real-time PCR, western blots and morphological data confirms the importance of macrophage polarization in stroke physiopathology [68,69]. In an experimental model of stroke, we found, that following ischemia/reperfusion the number of mononuclear cells is increased in the MZ of the spleen, and the Arg1/iNOS2 expression ratio on macrophages of marginal zone/red pulp interface is significantly shifted in the sham group compared to ischemic group in the favour of iNOS2 expressing cells (in press). Correspondingly, in the brain sections of the same animals we detected an overall reduced number of CD68-positive macrophages/microglia in the early (24h) infiltrates of ischemic cerebral tissue. These cells were present in a higher number in the penumbra than in the central core. The expression of cellular markers of macrophage polarization, iNOS2, and Arg1 was also higher in the penumbra than in the core and evidenced a significant a significant M1-phenotype dominance [70]. Thus, the modulation of microglia/macrophage polarization represent another promising therapeutic possibility for stroke.

## Conclusion

Acute cerebral ischemia triggers a prompt neuroinflammatory response involving resident microglia as well as peripheral immune cells. The various immune cells contribute significantly to both brain damage and repair processes making unfeasible a categorization of stroke-related inflammatory processes as either exclusively beneficial or detrimental. The spleen plays a central role in the coordination of inflammatory responses in stroke although the exact mechanisms underlying the splenic responses after stroke are not fully identified. The different subsets of splenic cells seemingly play distinct roles in different stages of the stroke especially through the release of various cytokines\chemokines affecting both the local and systemic inflammation after stroke. Moreover, stroke-induced immune response is increasingly recognized to influence the neuropathological outcome after ischemic brain injury. Thus, further studies focusing on the spleen-brain cross-talk with their variety of cells and signaling molecules already in the early stages of neuroinflammation in acute brain ischemia are necessary 1) to provide new insights into the role of immune response in the pathogenesis of ischemic brain injury and 2) to answer the question if in addition to the traditional surgery and thrombolytic therapy immunomodulatory interventions targeting the spleen could represent a complementary and wider therapeutic time window strategy, especially for improvement of the long-term prognosis in stroke patients.

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## Conflict of interes

The author has no conflicts of interest to declare.

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RESEARCH ARTICLE

# Evaluation of the Apical Seal after Root Canal Cleaning and Shaping with Two Nickel-Titanium Systems

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**Objective**: The aim of our study is to compare the ability of two nickel-titanium systems that use different rotation motions to create preparations that could promote a complete filling of the apical third of root canals. **Methods**: We used 36 freshly extracted teeth, randomly divided in two groups, as follows: in Group A we used ProTaper Next, a system characterized by a continuous rotary motion and in Group B the teeth were instrumented with Wave-One, in which the files have a reciprocating motion. All teeth were root filled based on the same protocol, using gutta-percha and AH Plus. The teeth were further prepared for microleakage evaluation based on dye penetration technique, as follows: immersion in 2% methylene blue, longitudinally sectioned and examination of the apical thirds with an operating microscope. The distance of dye penetration along dentin walls was measured using the ImageJ program. **Results**: The comparison between rotational and reciprocating systems showed that reciprocating files significantly promoted a reduced apical microleakage, as demonstrated by unpaired t test, Welch corrected (p=0.0346). **Conclusion**: The use of Wave-One Reciprocating system was considered more effective in the shaping of root canals, as they demonstrating better conditions for the hermetic, tridimensional sealing of apical third of the roots canals.

**Keywords**: apical microleakage, ProTaper Next, Wave-One, cleaning and shaping, dye penetration technique

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## Introduction

The endodontic therapy is performed in order to eliminate all inflammatory and/or infectious dental pulp tissue, with a final hermetic and tridimensional sealing of the entire root canal system, which was demonstrated to provide a high long-term success of the treatment. The correct application of the mechanical and biological phases of cleaning and shaping creates smooth dentin walls, facilitate irrigation and obturation, with concomitant emphasis on the preservation of the shape and location of the apical foramen. [1,2] These principles, introduced by Schilder in 1974, have undergone great transformation in recent years, due to the gradual replacement of the manual instruments by the rotary modeling systems and techniques, represented mainly by continuous movement and alternating reciprocating systems. [3]

During the last decade, many types of Nickel-Titanium (Ni-Ti) rotary files have been introduced on the market, such as ProTaper (Dentsply Maillefer, Ballaigues, Switzerland) and Wave-One (Dentsply Maillefer, Ballaigues, Switzerland). These instruments have an improved cutting efficiency and safety but on the other hand, their disadvantages are represented by the need to use several instruments, an increased fracture risk and cross contamination. [4] The reciprocating motion compared to continuous rotation could be advantageous regarding the reduced stress and time required for the preparation of curved canals, with a single use of a Ni-Ti file. [5] This motion proved to have important benefits: the file rotates at lower speed, the risks of cyclic fatigue and torsional failures are decreased and the treatment is more cost-effective. Wave-One files describe a contraclockwise (CCW) rotation of $170^0$ and a clockwise (CW) rotation of $50^0$ which means that it takes three reciprocating movements for the instruments to complete $360^0$. Therefore, this type of motion was considered more suitable for narrow or sclerotic canals. [6] This method of using only one file that works in reciprocating movement, performs rotations in CCW and CW direction, as follows: the instrument, when rotating in the cutting direction, advances inside the root canal, contacts the dentin and processes the cut, while when rotating in the opposite direction, it is released immediately. [7]

The continuous rotating instruments showed a greater tactile touch and efficiency when Ni-Ti files were used in smaller-diameter and more curved canals, which must be balanced with the risks associated with torque and cyclic fatigue failures. Fortunately, these disadvantages have been greatly eliminated after the continuous improvement in the file designs, use of high performance Ni-Ti alloy and more emphasis on the glide path management. In comparison to reciprocation, the well-designed Ni-Ti files used with continuous rotation, require less inward pressure and improve hauling capacity, pushing the debris out of the root canal. Current motors that drive reciprocating shaping files through equal forward and reverse angles generally require multi-file sequences for an adequate endodontic preparation. Furthermore, the systems that use equal CW/CCW small angles have also limitations, represented by decreased cutting efficiency, need for more inward pressure and low capacity to auger debris in coronal direction. The ProTaper Next system is a new generation of rotary instru-

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ments with Ni-Ti M-wire technology, which is the result of a thermo-mechanical processing that makes the instrument more flexible and resistant to cyclic fatigue compared to conventionally ProTaper files. [8]

Apical leakage is a well-known cause of endodontic failure, being influenced by many variables: different filling techniques, physical and chemical properties of endodontic sealer, presence of smear layer. [9] As microorganisms can survive inside dentinal tubules even after rigorous cleaning and shaping, the hermetical apical seal is desirable to block the remaining bacteria and their by-products and toxins from reaching the periapical tissues. [10, 11]

The aim of our study was to evaluate on extracted teeth, if the type of rotary Ni-Ti system used for endodontic preparation influences the quality of the root filling. The evaluation of the apical seal will be assessed using the dye penetration technique, a method commonly used for microleakage studies, due to its simplicity and cost-effectiveness. The null hypothesis to be tested is that the technique of root canal preparation has no influence on the quality of the endodontic filling.

## Methods

Our investigation was conducted after we obtain permission from the Ethics Committee of our university and in accordance to Helsinki Declaration. We used 36 freshly extracted human teeth, as part of an orthodontic treatment protocol, that were fixed in 10% buffered formalin solution before endodontic instrumentation. The teeth were randomly selected to the study groups and the endodontic instrumentation was carried out as follows: in Group A 18 teeth were prepared with the ProTaper Next rotary system (Dentsply Tulsa Dental, Oklahoma, USA) and in Group B other 18 teeth were instrumented with the Wave-One reciprocating system (Dentsply Maillefer Instruments SA, Ballaigues, Switzerland). After coronal access, a #10 file was introduced in each canal until it appeared at the apex and from this distance 1 mm was reduced, obtaining the working length. In Group A, initially the root canals of the specimens were explored with a #15 manual K-file, followed by instrumentation with the X1 and X2 files; the procedure was completed with X3 and X4 rotary files. The root canals were kept irrigated with a solution of 2.5% sodium hypochlorite throughout the preparation procedures, using copious amounts of solution after each instrument. In Group B, the root canals were first explored with a #15 K-file and then we used the Wave-One large reciprocating file (40/08) with an in-and-out movement. Likewise, the canals were irrigated with 2.5% sodium hypochlorite. After the biomechanical preparation of both groups, the canals were irrigated with 5 mL of EDTA solution 17%, in order to remove the smear layer. The final irrigation of root canals was performed with 10 mL of 2% Chlorhexidine solution and dried with paper points. For the root fillings we used gutta-percha and AH Plus (Dentsply Maillefer), using the continuous wave of condensation technique. The access cavities were cleaned of endodontic materials just below the cement-enamel junction and filled with glass-ionomer cement. All teeth were incubated at 37 C° 100% humidity for 7 days to allow the complete set of the sealing materials. For microleakage evaluation, all roots surfaces except the apical 3 mm were covered with two coats of nail polish and immersed in a 2% methylene blue dye for 24 hours days. The teeth were rinsed with running water and dried and on each root a buccal and lingual longitudinal groove was made, using diamond disks under water coolant, ensuring that the root canal remains intact. Then each root was split into two halves by levering with a knife. Color photographs were taken using a Sony a6000 camera and transferred to a personal computer. The maximum degree of dye penetration was recorded for each section and the degree of leakage was determined from cement-enamel junction to the coronal limit. The readings were made by two observers that were previously calibrated who used the ImageJ Computer Program in order to measure the extent of dye penetration. The data was submitted to statistical analysis using unpaired t test, Welch corrected ($p<0.05$).

## Results

The use of rotary Ni-Ti systems enabled a more predictable canal preparation, with few procedural errors, mainly in narrow, curved canals. The well-known limitation of these instruments, represented by the apically tendency to straighten the canal (zipping), was not observed in our study groups. On the specimens examined, the apical curvature of the canal was maintained and completely filled with endodontic sealer and gutta-percha. The images used for microleakage assessment are presented in Fig.1-3 and the statistical analysis is summarized in Table 1. The mean values of apical microleakage recorded after the use of Wave-One reciprocation technique and ProTaper Next rotary system are presented in Table 1. The use of a visible scale was an indication of the amount of vertical dye infiltration and the exact measurement was further obtained with the computer program. The statistical analysis of the recorded data showed that there is a significant difference

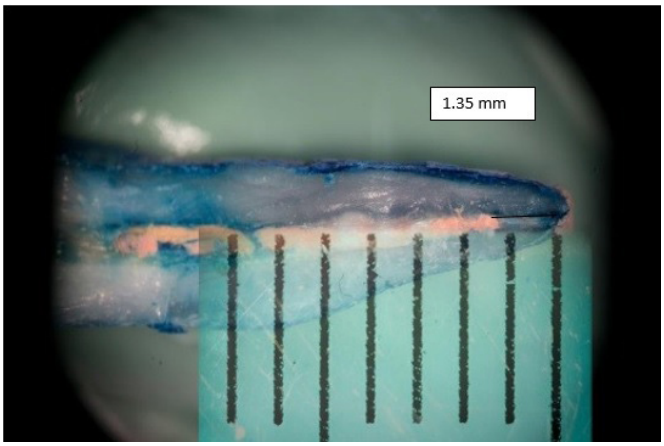


**Fig.1 A visual scale was used to illustrate better the value of vertical dye penetration, measured from the apical cement-dentinal junction into coronal direction (Specimen from Group A, 1.35mm).**

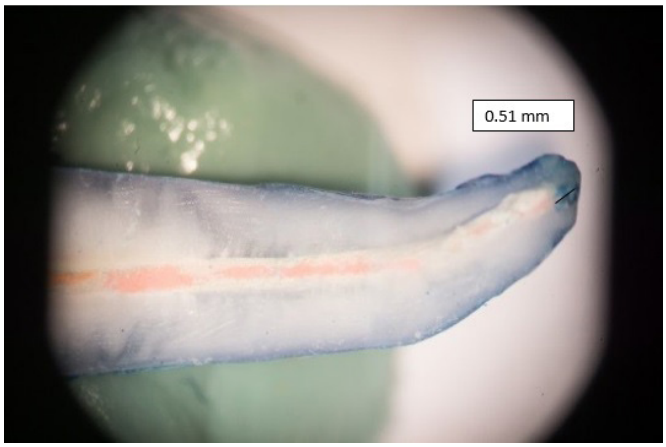


Fig.2 The use of Wave-One system (Group B) maintained the initial shape of the root canal and the apical infiltration of the dye was reduced (0.51mm).

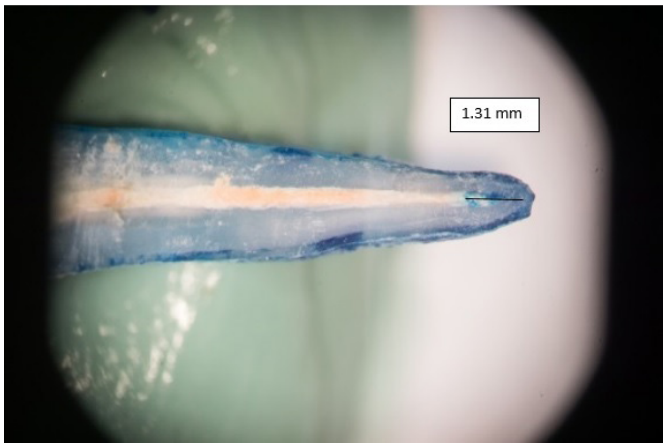


Fig. 3 Specimen from ProTaper Next (Group A), which showed more apical microleakage (1.31mm).

Table 1. The values of vertical apical microleakage recorded in the study groups, based on dye penetration technique

| Study group | Number of teeth | Minimal apical microleakage | Maximal apical microleakage | Mean | Standard Deviation |
|---|---|---|---|---|---|
| ProTaper Next | 18 | 1.43 | 1.96 | 1.617* | 0.193 |
| Wave-One | 18 | 0.51 | 1.83 | 1.482* | 0.174 |

*Statistically significant differences (p=0.0346, p<0.05)

between WaveOne and ProTaper Next regarding the apical seal, as reciprocating group produced the best apical seal (p=0.0346). Therefore, the null hypothesis was not confirmed.

## Discussion

Numerous studies have evaluated the apical sealing using different methods like dyes, radioisotopes, bacteria and their products or methodologies such as light microscopy and digitally captured images for evaluation of sealer-dentin interface. The prevention of apical leakage of root canal fillings implies the presence of a three dimensional apical and coronal sealing. Today, with the new materials and techniques available on the market, the potential of successful outcomes is higher, as these create a better seal between root canal walls and the filling material. Many studies focused on the ability of different Ni-Ti rotary instruments in shaping of simulated root canals or on extracted teeth; in our investigation we used the latter, as the first one is considered to have little relevance to the complex anatomy of the endodontic space.

In our study, we used the dye penetration method, to evaluate and compare the amount of apical microleakage in root canals filled with the same endodontic sealer but shaped with two different Ni-Ti rotary systems, ProTaper Next and Wave-One, which became very popular among dental practitioners, being also considered appropriate for undergraduate teaching. Their characteristics could be summarized as follows: the unequal bidirectional movement of Wave-One system, compared to continuous rotation of ProTaper Next, offers a significant improvement in safety, as the CCW engaging angle is smaller than the elastic limit of the file. Furthermore, different from other reciprocating systems that use equal bidirectional angles, the Wave-One system has an engaging angle which is 5 times the disengaging angle, so that after three engaging/disengaging cutting cycles, the file completes a 360º rotation. This enables the instrument to advance more readily to the working length and enhances auguring debris out of the canal, promoting the biological objectives of cleaning and also, shaping and proper filling of the root canal system. [6, 12] All phases of endodontic therapy are important for the final outcome, as any carelessness may compromise the long-term good results of this therapy. Among these, the biomechanical preparation of the root canal has a special place and a thorough knowledge on instruments used is absolutely necessary for a successful treatment. [13]

In our study, the use of Ni-Ti rotary instrument produced a well tapered root canal form, facilitating the filling and completing the preparation in an acceptable time, also maintaining the canal curvature. This finding is in agreement with observation of other studies and may be explained by the great flexibility of Ni-Ti files, which have a superior ability to follow even the severely curved canals. [14] Therefore, we used manual files only for root canal exploration prior to mechanical preparation, in order to assure a smooth glide path through the internal anatomy of the teeth. One of the advantages of the mechanized instrumentation is the promotion of a faster preparation of the root canal, with less stress for both dental specialists and their patients during treatment. The continuous rotary systems revealed in time their failures, leading to the development of a technique in which only the F2 file of the Protaper Universal system was used in all root canal instrumentation, based on reciprocating kinematics instead of continuous, in order to reduce the instrument fatigue and simplify the preparation steps. [15-17]

Many studies compared the effectiveness of the continuous versus reciprocating movements concluding that Ni-Ti instruments used with reciprocating movements had greater resistance and ability to maintain the initial course

of the canal, showed lower apical transportation and less apical extrusion of dentin, compared to continuous instrumentation. [18, 19] Proper enlargement of the root canal is essential, as microorganisms can penetrate the dentin tubules; furthermore, the pre-dentin must be completely removed during preparation to avoid formation of voids between the endodontic sealer and the root canal walls. Both systems are made from Ni-Ti alloy (M-wire), but they have different cross-sections: Wave One files section varies along their axis, while Protaper Next files have a decentralized rectangular cross-section that creates an enlarged space for removing debris and resulting in asymmetrical movement, where only two edges of the instrument come into contact with the canal wall. The use of large diameter instruments might compromise the root canal, creating morphological changes which can lead to treatment failure. [20] The conservative preparation of the apical third ends up compromising the cleaning of the root canal; accordingly, the diameter of the instruments used in the present study is consistent with the anatomy of the dental elements chosen. In our study, each specimen was instrumented with files of equal diameter, ensuring a correct and reliable standardization and comparability of the experimental groups.

A common method used to evaluate the value of apical microleakage is based on linear measurement of dye penetration. Eosin, Methylene blue, Procian brilliant blue, and Black India Ink are some of the dyes frequently used. In our study we used Methylene blue, as its molecular size is similar to bacterial by-products such as butyric acid, which can leak out of infected root canals to irritate the periapical tissue, it is easy to use and is available. It is an epoxy-bis-phenol resin based sealer that contains adamantine and has the ability to bond to the radicular dentin. However, AH Plus tends to shrink causing early debonding from the root canal wall, but it has a greater adhesion than Epiphany Pentron Clinical Technologies, Wallingford, USA), probably due to the fact that AH Plus has better penetration into the micro-irregularities. [20] The long setting time increases the mechanical interlocking between sealer and root dentin. On the other hand, there is inadequate bonding between the sealer and the gutta-percha point, allowing fluid leakage at this interface. We used the potential of this dye to flow through the apex, in order to determine the quality of endodontic shaping obtained by using ProTaper Next and WaveOne systems. Methylene blue dye showed a great potential to enter through the complex anatomy of the apical third or at the interface between dentin-sealer-core material interfaces. In our study we used AH Plus as the common sealer in both study groups, due to its radioopacity, biocompatibility, ease to use and availability, which determined many researchers to consider it the gold standard for endodontic research.

## Conclusion

Shaping ability of Ni-Ti rotary systems is of utmost importance for specialists in Endodontology, as these can influence the apical filling and finally, the treatment outcome. In the current study, the result of the use of rotary NiTi instruments with matched-tapered gutta-percha cones showed minor microleakage in term of linear dye penetration.

Within the limitations of our study, we can conclude that WaveOne with its reciprocating motion created better conditions for a perfect apical seal and preserved the initial shape of the root canal, without apical transportation. Nevertheless, one should take into consideration that this experiment was conducted in vitro, with its inherent limitations and therefore clinical extrapolation should be avoided.

## Authors' contribution

Amalia Abageru (Data curation; Methodology; Writing – original draft)
Mihai Pop (Formal Analysis; Methodology)
Monika Kovács (Supervision; Writing – original draft)
Alexandra Stoica (Data curation; Formal analysis)
Monica Monea (Conceptualization; Supervision; Validation; Writing –review & editing)

## Conflict of interest

None to declare.

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RESEARCH ARTICLE

# Clinical Study on the Perception of Patients with Orthodontic Appliances Regarding the Periodontal Change

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**Introduction**: The orthodontic treatment helps in reestablishing a functional occlusion, improving the aesthetics and functionality of the dento-facial complex. A correct alignment of the teeth, through the correction of some dental or skeletal anomalies, enhances the possibilities of dental hygiene, thus reducing the risk of periodontal affections. Nevertheless, the presence of orthodontic appliances in the oral cavity may reduce the efficacy of the means of oral hygiene by creating retentive areas for food, thus producing damages at the level of the marginal periodontium. **Objective**: The evaluation of oral hygiene practices for patients in the course of fixed orthodontic therapy and the identification of changes appeared at the level of the marginal periodontium caused by the orthodontic appliances.
Material and methods: A questionnaire with 20 questions was distributed to a number of 129 patients undergoing the active phase of orthodontic treatment in the Orthodontic and Dentofacial Clinic of the Medical Dental School Tîrgu Mureș or in some private practices in the Bucharest metropolitan area. **Results**: The majority of patients surveyed are aware of the means of dental hygiene and practice a daily brushing, associated with auxiliary means. The iatrogenic effects of the fixed therapy are represented by gingival recession, gingival overgrowth and bleeding during brushing but these were visible only in the case of a small number of patients. **Conclusions**: The fixed orthodontic treatment must be started only after a thorough evaluation of the marginal periodontium, with a close orthodontist – periodontist collaboration and avoidance of jiggling-like movements.

**Keywords**: questionnaire, orthodontic appliances, marginal periodontium

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## Introduction

Numerous studies prove the interrelation between orthodontic treatment and periodontal treatment at the level of the oral cavity. A correct alignment of the teeth and an adequate interarcadial relation help in improving the facial aesthetics or the masticatory efficacy. This enhances the health of the entire organism, thus raising the quality of life. A correct alignment of the teeth, through the correction of dental or skeletal anomalies, multiplies the possibilities for dental hygiene, thus reducing the risk of periodontal diseases [1, 2].

The orthodontic treatment helps to reestablish the functional occlusion, improving the aesthetics and the functionality of the dental-facial complex. It is well known that the dental malocclusion represent a contributing factor for the appearance of periodontal disease [3]. This comes in the third place regarding the pathology of the oral cavity. By correcting certain dental malpositions such as crowdings, diastemata, or proclinations, the risk of periodontal affection is reduced by relieving the oral hygiene and by directing the occlusal forces in the tooth's ax, thus ensuring their uniform distribution [4].

Considering all this, the presence of orthodontic appliances in the oral cavity may lead to the decrease of the means of oral hygiene by creating several retentive areas for food. Furthemore, the application of orthodontic appliances leads to the appearance of additional surfaces in the oral cavity, with different properties as compared to those of the natural oral surfaces. These contribute to the development of the biofilm and to the multiplication of cariogenic and periodontopathogenic bacteria [5]. Sometimes, the components of orthodontic appliances may produce lesions at the level of oral mucosa. Frequently, these lesions are located in the lingual or oral mucosa, or respectively in the retromolar area, where the lesion is produced by the extension of the arc towards the distal area. The lesion of the oral epithelium leads to the exposure of nerve endings, producing a sensation of pain and discomfort. The pain appears at an interval of approximately 24 hours from the moment of the lesion.In the absence of infections, the ulceration disappears in an interval of 12-14 days from the removal of the causal agent [6].

Thus, a rigurous oral hygiene is essential in maintaining an adequate periodontal status. Apart from the brushing frequency, an important role is played by the type of dental brushing, the patient's dexterity, and the adjunctive oral hygiene aids recommended by the specialist doctor (e.g., interdental brushes, mouthwash, dental floss). These need to be individually adapted based on the patient's dexterity, her motivation, or her predisposition to developing dental or periodontal affections. Together with accepting the or-

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thodontic treatment, the patient needs to be conscious of the importance of oral hygiene and of the changes brought by the dental hygiene practices.

The aim of this study is to evaluate the oral hygiene practices of patients in the course of fixed orthodontic therapy and to identify the changes that appear at the level of the marginal periodontium caused by these orthodontic appliances.

## Material and methods

A questionnaire with 20 questions was distributed to a number of 129 patients undergoing the active phase of the orthodontic treatment in the Orthodontic and Dentofacial Clinic of the Medical Dental School Tîrgu Mureș or in some private practices in the Bucharest metropolitan area. All patients included in the study are over the age of 13 and are undergoing fixed orthodontic treatment. The questionnaire was distributed during January – March 2019.

The questionnaire contains closed-answer multiple choice questions. The questionnaire is self-developed, with multiple choice options, phrased concisely and for the easy understanding of each patient. It was filled in the waiting room, in the presence of an examiner that was available to answer any unclarities regarding the questions. The time required to fill in a questionnaire was approximately 2 minutes.

The questionnaire is annonymous and contains questions structured in 4 parts. The first 5 questions (1-5) focus on the demographics and treatment period details. The following 6 questions evaluate the hygiene put in place by patients, the brushing methods and the time allocated for brushing (6-11). The following 4 questions (12-15) contain information about the adjunctive oral hygiene aids used, the frequency with which these are used, with the role of observing the patients' compliance and the doctor's attitude in what regards them informing patients about the existence of such adjunctive methods. The last 5 questions (16-20) refer to the changes which appeared at the level of the marginal periodontium following the application of orthodontic devices.

Informed consent was obtained from all patients or from their parents/guardians.

The obtained results were analysed statistically using the Chi square test.

## Results

129 patients were surveyed, 73 of which were women. The 13-17 years old segment represents 43% of all patients, 32% of patients being in the 18-25 years old segment, while 26% of them are over 25 years old. The majority of patients comes from the urban areas (64%). The majority of patients (45%) had been undergoing treatment for 6-12 months, 33% of patients were at the beginning of the orthodontic treatment (below 6 months), while 19% of patients had been undergoing treatment for longer than 1 year.

There is a statistically significant correlation between the area of residence (urban / rural) and the type of dental clinic visited. Thus, only 12% of patients coming from urban areas chose to follow a treatment in a state clinic, while 28% of patients in the rural area selected such clinics (p value : 0.0422)

The manual toothbrush is the most used method of oral hygiene (58.59%), while 23.44% of patients use the electric toothbrush, and 17.97% use both types of brushes. Furthermore, 11% of patients coming from rural areas use the electric toothbrush as compared to 39% of patients from urban areas (p value : 0.0014). Regarding the softness of the brush used, the most prevalent type is the soft bristles toothbrush (35%), ultra soft bristles (29%), or medium bristles (34%), while hard bristles are only used in 3% of cases. Female patients are mostly using manual toothbrushes with ultra soft bristles (39%), while male patients use the toothbrush with soft bristles (37%) (p value: 0.0238).

Fig. 1 presents the methods of brushing used by the patients. They generally undergo a vertical brushing (55%), or a combined style of brushing (24%).

Only 5% of patients have not noticed any changes in the habits of oral hygiene (changes in the duration or frequency). 53% of patients observed both increases in the frequency and in the duration of the brushing, 30% observed increases in the duration of the brushing, while 12% observed increases in the frequency of brushing. The

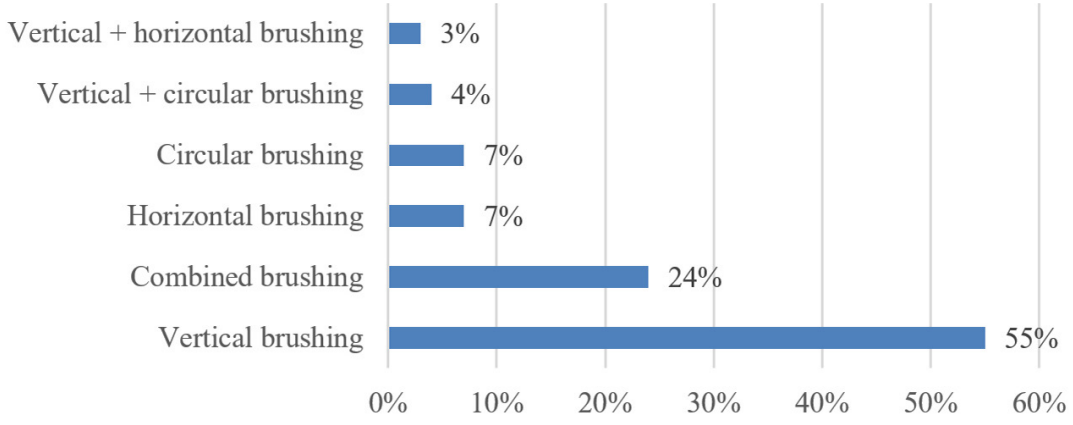


Fig 1. Distribution of patients by type of brushing

majority of patients undergo a regular brushing, 2 or more times per day (45% and 48%, respectively), a significantly higher percentage compared to the patients who undergo a 3-5 min long brushing (57%). Female patients brush 3 or more times per day (61%), while male patients predominantly brush 2 times per day (55%) (p: 0.0044). (Fig.2)

Concerning the awarenesss on the adjunctive oral hygiene aids, the majority of patients (91%) were informed regarding their use, 2% declared they were not informed, while 6% answered inconclusively „I don't know". The frequency of use of the adjunctive oral hygiene aids is represented in Table I.

We also observed a statistically significant correlation between use of mouthwash and the patients' age. The 13-18 years segment mouthwash is used 2 times per day (43%), while the rate of heavy users is decreasing significantly with the increase in age (p: 0.0474).

The frequency of gingival changes induced by the presence of orthodontic appliances is represented in Tablel II. 10% of patients did not observe any change in terms of gingival bleeding as compared to the period prior to the start of the orthodontic treatment.

## Discussions

The periodontal status of the patients undergoing orthodontic treatment present an interest for both the orthodontist and the periodontist. Any misbalance that appears at the periodontal level is felt by the patient either through bleeding, tumefaction, changes in the gingival appearance, or sensitivity at contact with different stimuli. These can be perceived either as disconfort or can create significant pain which demotivates the patient or may compromise the entire treatment.

In our study, the patients were relatively uniformly distributed by gender (73 women and 56 men), with the majority coming from urban areas (64.34%). The patients from rural areas prefer to undergo treatments in state clinics in a higher rate (28%) as compared to urban area patients who prefer private clinics (88%) (p: 0.04).

For a more efficient removal of dental plaque, several manual or electric devices were created that, used together with antibacterial substances offer favorable results in reducing gingivitis. Numerous studies compare the efficiency of the manual brush compared to the electric one, yielding mixed results. A single study demonstrated a better efficiency of the manual brush in removing dental plaque as compared to the electric brush [7].

Other authors, on the other hand, did not find a difference between the efficacy of the two types of brushes [8,9]. Of all patients included in our study, 58.59% use the manual toothbrush as the main method of oral hygiene, using a vertical brushing (55%) or a combination of the vertical, horizontal, and circular movements (24%). The electric toothbrush is used to a lower extent (24.44%) while an even lower number of patients use both types of brushes alternatively (18%). We also observed the fact that the electric toothbrush is used more by patients from urban areas (p: 0.0014). Very few patients from rural areas (19%) use the electric toothbrush, probably due to the more difficult access to new technologies and a weaker purchasing power compared to the urban areas. The patients included in our study are well informed regarding the consistency of the manual toothbrush and its efficacy. Only 3% of patients use the manual toothbrush with hard bristles. The ultra soft bristles toothbrush is used in particular to obtain a finesse brushing of the gingival sulcus or in case of a high dental sensitivity. This brush is used by the patients in the study in a lower rate (29%) and is used particularly by female patients (39%) vs. male patients (14%) (p: 0.0238). Using supporting oral hygiene methods, such as toothbrushes

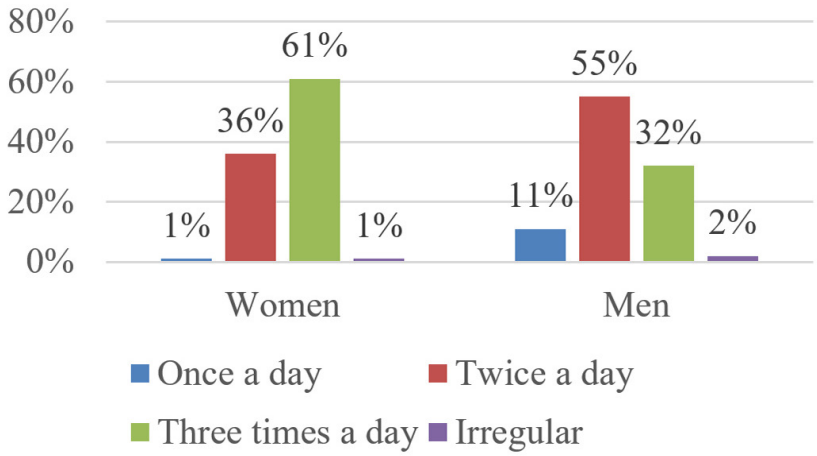


Fig 2. Correlation between gender and brushing frequency

Table I. Frequence of using auxiliary oral hygiene methods

| | Once / day | Twice / day | 3 times / day | Never | Total |
|---|---|---|---|---|---|
| Mouthwash | 45% | 34% | 9% | 12% | n:128 |
| Interdental brush | 40% | 34% | 18% | 8% | n: 128 |
| Super Floss | 45% | 13% | 5% | 37% | n:129 |

Table II. Frequency of changes appeared at gingival level

| | Yes | No | I don't know |
|---|---|---|---|
| Bleeding (n:116) | 16% | 63% | 11% |
| Gingival overgrowth (n:129) | 12% | 66% | 22% |
| Sensitivity (n:129) | 46% | 46% | 8% |
| Ulcerations (n:129) | 23% | 62% | 15% |
| Gingival recession (n:129) | 4% | 65% | 31% |

dedicated for patients with fixed orthodontic appliances, can lead to an increase in the brushing time as compared to the time allocated for brushing before the beginning of treatment. The patients surveyed for this study observed a change in the methods of oral hygiene. The majority has observed an increase in the time allocated for brushing, but also an increase in the frequency. Female patients showed a higher interest for oral hygiene (p: 0.0044), brushing 3 or more times a day (61%) as compared to male patients who brush twice per day (55%).

In our study, we noticed a more frequent use of adjunctive oral hygiene aids. The interdental brush was the most used method of auxiliary hygiene (91.40%), used predominantly once (40%) or twice per day (33.60%). The mouthwash is used by 87.5% of patients, mostly once per day (44.5%). The super floss is the least used method of auxilliary hygiene (63%), but also holds a rather high frequency. A similar study undertaken in India on a lot of 40 patients aged 18-30 years old, shows that 2% of patients use super floss, while 7% of them use interdental brushes. 42% of patients use mouthwash with 24% of them doing it once per day and 18% twice per day [10]. Also in India, Mayuresh J Baheti and his collaborators, in a study on 150 patients aged 13-20 [11], observed a use of interdental brush for 22.60% of patients, while mouthwash was used by 31.33%. Similar results were obtained in a study undertaken by Priscila Ariede and collaborators in Brasil, where 11.11% of the 27 volunteers included in the study use super floss and interdental brushes [12].

The different results of the three studies compared to our results is probably due to the significantly different social and/or economic status of the countries involved in the study. In our work, we observed a correlation between patients' age and use of mouthwash.The majority of patients use mouthwash (87.5%), particularly in the 13-17 years old segment, who use mouthwash two or more times a day. Once the age increases, the use of mouthwash decreases significantly (p: 0.047).

The number of patients who observed the appearance of a dental sensitivity is almost equal to the number of patients who did not observe something similar (60 and 59 patients, respectively). Although the dental sensitivity has been reported by almost half of the patients, very few noticed the appearance of gingival recession (only 5 patients did, while 40 patients responded inconclusively). In this case, the dental sensitivity may be induced by stripping, a method used to create space in order to avoid extractions. Furthermore, 80% of patients who denied the appearance of gingival recessions are 13-17 years old, with the possibility that the presence of a gingival recession may not have been correctly appreciated.

In the study conducted by Mayuresh J Baheti and collaborators [11] 52.77% of patients did not notice any change at the gingival level, considering it in a good state. 34.16% of patients classify the gum as being in a good state, while 13.05% of them consider it is in a state of suffering since the beginning of the orthodontic treatment. These parameters were also evaluated by Azaripour A and collaborators [13]. The gingival changes that refer to tumefaction, bleeding and changes in the colour of the gum are comprised into one question, to which 56% of patients responded affirmatively. In our study, 62.79% of patients denied the appearance of gingival bleeding, while 10% of them did not notice any change in the gingival bleeding as compared to the period prior to the orthodontic treatment.

Although the increases in the gingival volume often represent an iatrogenic effect of the fixed orthodontic treatment, only 12% of patients included in this study noticed the presence of gingival hiperplasia / hypertrophy. The increases in the gingival volume most often appear in cases of patients with precarious hygiene or in cases of prolonged orthodontic therapy [14]. The low frequency of the gingival volume increases noticed by patients included in our study is due to the correct hygiene practiced and to the short period of orthodontic treatment, which exceeds 24 months only in the case of 19% patients.

In our study, a relatively small number of patients signaled the appearance of ulcerations in the oral mucosa (24% answering affirmatively, 15% answering inconclusively), as compared to other studies in which patients signaled the presence of ulcerations to a higher degree. 95% of patients surveyed by Kvam E and collaborators observed ulcerations in the oral mucosa [15]. However, 83% of them labeled this inconvenience as a minor one. In order to prevent the appearance of ulcerations, the curating doctors of the patients included in this study recommend the use of protective wax in the respective areas.

## Conclusions

1. The orthodontic therapy of adult patients will start after the evaluation of the periodontal status, while for patients that have suffered from periodontal issues, an antiinflamatory therapy and a new evaluation of periodontal indices PI (plaque index), BOP (bleeding on probing), CAL (clinical attachment level) is recommended before the application of the fixed orthodontic appliance.
2. The majority of surveyed patients are aware of the dental hygiene methods and use a daily vertical brushing, associated with adjunctive oral hygiene aids, for around 3-5 minutes.
3. Female patients brush more frequently, 3 or more times per day, as compared to male patients that brush around 2 times per day.
4. The iatrogenic effects of the fixed therapy for adult patients are represented by the gingival recession, bleeding during brushing and hyperplasia, but these were only present for a small number of patients.
5. The gingival recession and hyperplasia are destructive processes that may also appear in the absence of orthodontic treatment, which makes the anamnesis

important in signaling risks factors prior to applying orthodontic forces.

6. The fixed orthodontic treatment for adults must be instituted only after the rigurous evaluation of the marginal periodontium, with a close orthodontist – periodontist collaboration, and by avoiding the jiggling-like movements (that overstress the muco-gingival junction).

## Abbreviations

PI- Plaque index
BOP- Bleeding on probing
CAL- Clinical attachment level

## Conflict of interest

None to declare.

## Authors' contribution

Simina Chelărescu (Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Writing – original draft)
Karina Dombi (Data curation)
Oana Gânscă (Data curation)
Panainte Irinel (Funding acquisition)
Olteanu Cristian (Software)
Mariana Păcurar (Supervision; Validation; Visualization; Writing – review & editing)

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## Appendix - Questionnaire

Perception of patients with orthodontic appliances regarding the periodontal changes

1. Age
   a. 13-18 years
   b. 18-25 years
   c. > 25 years

2. Gender
   a. Female
   b. Male

3. Background
   a. Urban
   b. Rural

4. Type of dental clinic
   a. Private dental clinic
   b. Public dental clinic

5. When did you start the orthodontic treatment?
   a. < 6 months ago
   b. 6-12 months ago
   c. > 12 months ago

6. What type of toothbrush do you use?
   a. Manual
   b. Electric
   c. Both

7. What type of bristles do you use?
   a. Extra soft
   b. Soft
   c. Medium
   d. Hard

8. What is your brushing tehnique?
   a. Vertical
   b. Horizontal
   c. Circular
   d. Combined

9. How did the orthodontic treatment interfere with the oral hygiene habits?
   a. Increase in toothbrushing time
   b. Increase in toothbrushing frequency
   c. Both of the above mentioned
   d. It did not influence me

10. How often do you brush your teeth?
   a. Once daily
   b. Twice daily
   c. Three times a day or more
   d. Irregular

11. How long do you brush your teeth for?
   a. 1-2 minutes
   b. 3-5 minutes
   c. 5-10 minutes
   d. Over 10 minutes

12. Have you been informed about adjunctive oral hygiene aids and how to use them?
   a. Yes
   b. No
   c. Not sure

13. Do you use mouthwash?
   a. Once daily
   b. Twice daily
   c. More than twice daily
   d. Never

14. Do you use interdental toothbrush?
   a. Once daily
   b. Twice daily
   c. More than twice daily
   d. Never

15. Do you use dental floss?
   a. Once daily
   b. Twice daily
   c. More than twice daily
   d. Never

16. Has the orthodontic treatment resulted in gingival bleeding?
   a. Yes
   b. No
   c. Not sure
   d. No change

17. Has the orthodontic treatment resulted in gingival enlargement?
   a. Yes
   b. No
   c. Not sure

18. Has the orthodontic treatment resulted in tooth sensitivity?
   a. Yes
   b. No
   c. Not sure

19. Have you noticed gingival recession? (change in the position of the gums, in the opposite direction of the tooth)?
   a. Yes
   b. No
   c. Not sure

20. Has the orthodontic treatment resulted in painful lesions?
   a. Yes
   b. No
   c. Not sure

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RESEARCH ARTICLE

# Fatal Road Traffic Accidents in Mureș County, Romania – A Retrospective Autopsy Based Study

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**Objective**: The main objective of this study is to evaluate the medico-legal aspects of fatal road traffic accidents. **Methods**: This is a retrospective study consisting of 80 forensic autopsies performed at the Institute of Legal Medicine – Tîrgu Mureș, Romania during a two years period, between January 1st, 2016 to December 31st, 2017. The information obtained was based on the medical records and the evaluation of autopsy reports. **Results**: Male victims involved in road traffic accidents were nearly three times more numerous than women (72.5% vs. 27.5%). Divided into 3 age groups (under 35 years old, 36-59 years old and over 60 years old) we noticed a relatively uniform distribution of the victims, with a slight dominance of the 36-59 age group and the over 60 years of age group. The highest number of victims was among the pedestrians (36.25%), followed in decreasing order by the drivers (33.75%), passengers (17.5%), cyclists (7.5%) and motorcyclists (5%). Positive alcohol testing was found in 14 of drivers (81 %). The analysis of lesions found during necropsies of the deceased at the scene of the accident highlights some particularly life-incompatible injuries that resemble any rescue effort on the part of the medical crew moved to the scene of the accident. **Conclusion**: Inappropriate road conditions and indiscipline in traffic of both drivers and pedestrians contribute to unacceptably high mortality.

**Keywords**: fatal road traffic accidents, autopsy, Mureș County

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## Introduction

One of the major preventable public health problems worldwide is the road traffic accident (RTA) which is on the rise and can be attributed to the increasing number of vehicles, lifestyle changes, and high-risk attitudes. When referring to global mortality, fatal RTAs account 1.7%, and 91% of the world's fatalities on the roads occur in the low and middle-income countries [1]. By 2020, road traffic injuries are expected to take third place in the rank order of disease burden [2]. According to the World Health Organization (WHO), in 2010, 1.25 million persons died in road traffic accidents all over the world; that is a life lost every 25 seconds [3]. The last WHO report, in 2018, shows that the problem is getting worse. The number has increased to 1.35 million a year, meaning nearly 3 700 deaths/day [4].

The widely used term, 'accident', according to the 1956 definition, can suggest an inevitable and unpredictable situation – an event that cannot be avoided. Contrary, in 2004 WHO report on Road Traffic Injury Prevention, the term "crash" is preferred [5].

RTAs are considered to be part of the "development diseases" and usually occur as a result of the increase in the number of motor vehicles, population densities, environmental changes and pollution [6]. Road traffic accidents are a major but neglected public health problem that is associated with high rates of mortality and morbidity worldwide [7]. The objectives of this study are to evaluate the medico-legal aspects of fatal RTAs, by researching the nature, type, and distribution of the traumatic lesions and establishing the most fatal injured body part.

## Materials and Methods

This is a retrospective study consisting of 80 forensic autopsies performed at the Institute of Legal Medicine – Tîrgu Mureș, Romania, during a two years period, from January 1st, 2016 to December 31st, 2017. The information obtained was based on the medical records and on the evaluation of autopsy reports.

## Results

From the total of 1802 forensic autopsies conducted in the study period, 80 (4.43%) were carried out for fatal road traffic accidents. In 2016, the total number of autopsies performed was 903 from which 44 (4.87%) for RTAs. In 2017, from a total number of 899 autopsies, 36 (4%) autopsies were carried out for RTAs.

From all the forensic autopsies performed for fatal RTAs, 58 (72.5%) were males and 22 (27.5%) were females.

The distribution by age group is relatively uniform: 24 (30%) cases under 35 years old and each 28 (35%) cases for the age group 36-59 years and over 60 years.

Among the categories of all traffic participants, pedestrians where the most involved and vulnerable in fatal RTAs, with a total number of 29 deaths (36.25%), followed by drivers with 27 deaths (33.75%), car passengers with 14 deaths (17.5%), bicyclists with 6 (7.5%) and with 4 deaths (5%) motorcyclists.

The majority of the subjects involved in RTA have died at the scene of the accident 44 (55%), followed by 18 (22.5%) which died one week or more after hospitaliza-

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tion, followed by 8 cases (10%) with 2-7 days survival; in 7 cases (8.75%), death occurred within the first 24 hours and in 3 cases (3.75%) death occurred during transport to a medical facility.

Depending on the categories of traffic participants, the highest proportion of deaths occurred at the scene of the accident: 75% of motorcyclists, 64.28% of passengers and 55.17% of pedestrians. Vehicle occupants died at the scene of the accident in a proportion of 65.85% (27 cases). From the group which was hospitalized over 1 week, 8 (66.66%) died due to infectious complications; from 3 victims with over 1 week of hospitalization organs for transplantation were prelevated. Most pedestrians, 20 cases (68.96%) died at the scene at the accident or in the first 24 hours; 8 (88.88%) of 9 patients with over 1 week of hospitalization died due to infectious complications.

Alcohol consumption among the victims was more frequently in pedestrians with 6 cases (20.68%), cyclists with 1 case (20%) and drivers with 4 cases (14.81%), with official measured alcohol values between 0.8 mg‰ to 1.4mg‰ for the involved drivers and up to values of 2.4 mg‰ for pedestrians (Table I).

Referring to the distribution of fatal accidents during the year, we observe a higher proportion in winter months (December-February): 11 driver deaths (45.83%) and pedestrians 24 deaths (82.75%). Accidents involving cyclists or motorcyclists prevailed in the spring-summer seasons with 3 cases (75%) for motorcyclists and 5 cases (83.33%) for cyclists.

Depending on the severity of the injuries that could have resulted in death, lesions of the cephalic extremity, including craniocerebral trauma (CCT) and craniofacial trauma (CFT) was found in 27 of the cases (33.75%), followed by polytrauma lesions in 25 of the cases (31.25%), lesions of major vessels 12 (15%), spinal cord transections 7 (8.75%), severe thoracic trauma in 6 cases (7.5%) and abdominal injuries in 3 deaths (3.75%) (Table II).

From all of the deaths recorded, in 16 cases (20%) there were no recorded lesions in the cephalic area, in the rest of the cases studied, 64 (80%) head trauma was present as follows: in 36 cases (45%) intracranial lesions where also associated with skull fractures; in 21 cases (26.25%) we did not identify skull fractures, but associated intracranial hemorrhagic manifestations were found; and in 7 cases (8.75%) we found skull fractures without hemorrhagic events (Table III).

From the distribution of the main lesions encountered in the topographical areas, we noticed that the lesions of the cephalic extremity have been observed at 64 victims (80%), followed by lesions resulting from thoracic trauma in 62 cases (77.5%), abdominal lesions with 33 cases (41.25%), heart and major vessels ruptures in 27 cases (38%) and spinal cord transections in 10 cases (27.02%).

Analysis of severe injuries capable of causing the death of victims at the scene of the accident shows that many victims have suffered heart and major vessels ruptures (52.27%), severe CCT (68.18%) with or without the evisceration of the brain tissue, cervical spine transections or of the cerebral trunk (38.63%) and thoracic trauma (52.27%).

Among the causes of death occurred in more than a week of hospitalization, deaths through infectious complications draw attention (98.44%), bronchopneumonia being the main complication found at the autopsy. Purulent leptomeningitis and meningoencephalitis, cerebral abscess and cardiogenic shock were also noted. Each time these complications were associated with severe injuries (Table IV).

**Table I. Distribution by gender, age, death interval and alcohol consumption**

| Road user | Gender | | Age | | | Death on site | Death on the way to hospital | Death <24 h | Death in 2-7 days | Death over 1 week | Alcohol Test (+) | Alcohol Test (-) | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Male | Female | <35 years | 36-59 years | ≥60 years | | | | | | | | |
| Driver | 24 (88.88%) | 3 (11.12%) | 8 (29.62%) | 10 (37.03%) | 9 (33.33%) | 14 (51.85%) | 1 (3.70%) | 4 (14.81%) | 4 (14.81%) | 4 (14.81%) | 4 (14.81%) | 23 (85.19%) | 27 |
| Passenger | 7 (50%) | 7 (50%) | 4 (28.57%) | 6 (42.85%) | 4 (28.57%) | 9 (64.28%) | - | - | 1 (7.14%) | 4 (28.57%) | - | 14 (100%) | 14 |
| Motorcyclist | 4 (100%) | - | 3 (75%) | 1 (25%) | - | 3 (75%) | - | 1 (25%) | - | - | - | 4 (100%) | 4 |
| Cyclist | 6 (100%) | - | 4 (66.66%) | - | 2 (33.33%) | 2 (33.33%) | - | - | 2 (33.33%) | 2 (33.33%) | 1 (20%) | 5 (80%) | 6 |
| Pedestrian | 17 \|(58.62%) | 12 (41.38%) | 5 (17.24%) | 11 (37.93%) | 13 (44.82%) | 16 (55.17%) | 2 (6.89%) | 2 (6.89%) | 1 (3.44%) | 8 (27.24%) | 6 (20.68%) | 23 (79.32%) | 29 |
| Total | 58 (72.5%) | 22 (27.5%) | 24 (30%) | 28 (35%) | 28 (35%) | 44 (55%) | 3 (3,75%) | 7 (8,75%) | 8 (10%) | 18 (22,5%) | 11 (13,75%) | 69 (86,25%) | 80 |

**Table II. Dominant lesions responsible for death**

| Type of injury | Number of cases | Percentage |
|---|---|---|
| CCT/CFT | 27 | 33.75% |
| Polytrauma | 25 | 31.25% |
| Major vessel injury | 12 | 15% |
| Spinal cord transection | 7 | 8.75% |
| Thoracic injuries | 6 | 7.5% |
| Abdominal injuries | 3 | 3.75% |

**Table III. Distribution of bone fractures and hemorrhagic lesions of the skull**

| Type of head trauma | No. of cases | Percentage |
|---|---|---|
| Skull fractures +hemorrhage | 36 | 45% |
| Hemorrhage, no skull fracture | 21 | 26.25% |
| Skull fracture, no hemorrhage | 7 | 8.75% |
| No hemorrhage, no skull fractures | 16 | 20% |
| Total | 80 | 100% |

Table IV. The main causes of death in victims hospitalized over one week

| Cause of death | Main lesion | No. of cases |
|---|---|---|
| Bronchopneumonia-septic shock | Polytrauma | 6 |
| Bronchopneumonia, septic shock | CCT, CFT | 4 |
| Purulent leptomeningitis, septic shock | CCT<br>Polytrauma with CCT | 1<br>1 |
| Purulent meningoencephalitis | CCT | 3 |
| Cerebral abscess | CCT | 1 |
| Bronchopneumonia | Abdominal trauma with liver/spleen ruptures | 1 |
| Cardiogenic shock | Polytrauma | 1 |

## Discussion

Romania is ranked first in a ranking of the European Union (EU) countries in terms of road fatalities, when considering the number of victims to the total population, according to European Commission data. In Romania, 1951 persons died in road traffic accidents in 2017, 2008 was the blackest of the last 17 years, with 3.065 deaths [8]. Within the European Union, Sweden, the United Kingdom, the Netherlands and Denmark reported in 2017 the lowest figures, with 25, 27, 31 and respectively 32 deaths/million inhabitants. On the opposite side, Romania has the most, 98 deaths/million inhabitants, which is twice the EU average (49 cases), followed by Bulgaria with 96. Romania and Bulgaria were the only EU countries where the report indicates over 80 dead to one million inhabitants [4]. The number of fatal road accidents in Romania recorded a slight increase in 2017 compared to 2016 [9,10]. According to the Romanian Criminal Investigation and Crime Prevention Institute and the Romanian Police Road Directorate, the main causes responsible for over 40% of the serious road accidents produced in Romania during the period 2013-2017 are pedestrians' inadequacies and excessive or inadequate speed to conditions traffic [10]. In our study, male victims involved in RTA were nearly three times more numerous than women (72.5% vs. 27.5%), the percentages were similar to other studies and explained by the male's social status in developing countries, male being the more active person [11-14]. Divided into 3 age groups, we noticed a relatively uniform distribution of the victims, with slight dominance of the 36-59 age group (passengers 42.85% and drivers 37.03%) and over 60 years of age group (pedestrians, 44.82%), making our data slight different compared to other reported data [11,15]; the first-mentioned group includes the majority of the socio-professionally active persons, which could explain the higher number of the victims being car occupants (driver/passenger), and in the group over 60 years the pedestrians predominate, probably due to lack of physical abilities and the deviations from the traffic safety rules, favored by the precarious condition of the local roads (lack of sidewalks and markings, insufficient lighting in the cities and absent outside them, often narrow runways and crowded). According to the Ministry of Internal Affairs (MIA), there are three main causes of serious road accidents recorded between 2015 and 2017: pedestrian carelessness, speed (inadequate to road conditions or illegal) and failure to grant pedestrian crossing [9]. The small number of cyclists/motorcycle victims (6 and 4 cases respectively) does not involve statistical discussions; however, the majority of the victims are part of the age group up to 35 years, as suspected, because these means of locomotion are mainly used by the young population. In our study, the highest number of victims is among the pedestrians (36.25%), followed in decreasing order by the drivers (33.75%), passengers (17.5%), cyclists (7.5%) and motorcyclists (5%), as the aforementioned report of the MIA also states [9]. We highlight the much higher percentage of pedestrian victims in our study, 36.25% compared to the EU average of 21% [4]. The casualties among drivers together with passengers (51.25%) are the most numerous, surpassing the number of pedestrian victims (36.25% as mentioned above), also corresponding to a survey conducted by the Emergency Medical Services (EMS) in Tîrgu Mureș, but for a different period [16]. The WHO data for 2016 show that in road accidents over the EU, a high number of pedestrians are killed: in 2016, nearly 21.2% of all road fatalities were pedestrians. This rate is considerably variable between countries: from less than 8.3% in the Netherlands to above 35% in Estonia and Romania. Finally, while in the EU the pedestrians` fatalities are calculated at 10.8 deaths per million inhabitants, in Romania, Latvia and Lithuania the statistics show a 3 times higher number [17]. These data are close to our statistics referring to Mureș County. Positive alcohol testing was found in 14.81 % of drivers and was more common in pedestrians (20.68%) and bicyclists (20%). The fact that accidents with victims among drivers (45.83%) and pedestrians (82.75%) predominate in the winter months (December to February) is correlated with the main causes of their production, like excessive speed, lack of speed adaptation to driving conditions, inadequate road conditions for drivers, crossings through unmarked places and the use of the roadside for pedestrian traffic. Accidents involving bicyclists or motorcyclists were predominant in the spring-summer seasons, 75% for motorcyclists and 83.33% for bicyclists, due to the specificity of these means of travel, used predominantly in the warmer months. It is known that road traffic is higher in the summer months and especially in holiday months; the fact that this interval does not correspond to the peak of the accidents in our study, makes us believe that the inadequate condition of the roads contributes mainly to the occurrence of accidents and it may be the reason for Romania's ranking in 2017 in the first place in the European Union in the number of deaths in road accidents [4,11]. Between 2016 and 2017 there were 3864 road deaths in Romania, the 80 victims in Mureș County accounting for 2.07%. For the year 2016 in Romania, the average annual deaths per county was 46.66 compared to 44 in Mureș county and in 2017 was 47.58 versus 36 in Mureș county; we,

therefore, ascertain a positioning of Mureș county below the annual average, with a decreasing trend without having noticed significant improvements in the state of the roads [10].

The highest mortality was recorded in the pre-hospital, 58.75% of which 55% at the accident site and 3.75% on the way to the hospital, the remaining 41.25% of the deaths occurred in the hospital, which corresponds to other data from the literature when referring to pre-hospital deaths, but in our statistics, hospital deaths are significantly higher [11,18], probably suggesting a hospital incapacity in assessing complex cases of polytrauma.

The analysis of lesions found necroptically in the deceased at the scene of the accident, highlights some particularly life-incompatible injuries that resemble any rescue effort on the part of the medical crew moved to the scene of the accident, with: large vessels ruptures (52%), CCT and CFT with evisceration of the brain, cerebral trunk sections, massive intracranial hemorrhages (68.18%), abdominal trauma with multiple organ ruptures (liver, spleen, kidneys) or thoracic injuries with pulmonary rupture and asphyxia by obstruction of the airway with blood. Classification on anatomical regions with death-related injuries is more conventional (didactic) because in many cases multiple lesions are each capable of producing death.

Analyzing the causes of the deaths of hospitalized victims following serious injuries in road accidents, we notice that when hospitalizations extend over a week, deaths mostly result from infectious complications (98.44%), which should draw attention to treatment options for major trauma.

## Conclusion

Romania has been in the top places in the EU for mortality by road traffic accidents for many years. Inappropriate road conditions and indiscipline in traffic of both drivers and pedestrians contribute to unacceptably high mortality. The lack of driving experience did not prove to be a favorable factor in producing fatal accidents, our data showing a higher death incidence in drivers over 35 years. Mortality at the scene of the accident is comparable to other EU countries and is due to life-threatening organic injuries and in no case to the incapacity of emergency crews. In-hospital mortality is much higher compared to other data in the literature, which denotes a deficiency of the medical system to assist large traumatized patients, with in-hospital infections being the leading cause of death. Road traffic accidents are a serious public health problem, not only considering the number of victims among drivers but also the collateral victims involved.

## Authors' contribution

Cosmin Carașca - Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing

Timur Hogea - Data curation; Formal analysis; Software

Viorel Hădăreanu - Supervision; Validation

## Conflict of interest

None to declare.

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RESEARCH ARTICLE

# Atomoxetine and Duloxetine: Evaluation of a Potential Pharmacokinetic Drug-Drug Interaction

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**Objective**: The present research aimed to investigate whether a pharmacokinetic drug interaction exists between atomoxetine, a substrate of CYP2D6 and duloxetine, an enzymatic inhibitor of the same metabolic pathway. **Methods**: Twenty-three healthy volunteers were enrolled in an open-label, non-randomized, sequential, 2-period clinical study. During the trial, they received a single dose of atomoxetine 25 mg (Period 1:Reference) followed by a combination of atomoxetine 25 mg and duloxetine 30 mg, after a pretreatment regimen with duloxetine 30-60 mg/day for 4 days (Period 2:Test). The pharmacokinetic parameters of atomoxetine and its main metabolite (4-hydroxyatomoxetine-O-glucuronide) were estimated using a non-compartmental approach and statistical tests were used to compare these parameters between study periods. **Results**: A total of 22 subjects, extensive metabolizers (EMs), were considered for the final report of the study findings. Duloxetine influenced the plasma concentration-time profile of both parent drug and its glucuronidated metabolite. The pharmacokinetic and statistical analysis revealed that pretreatment with the enzymatic inhibitor increased the mean atomoxetine $AUC_{0-t}$ (from 1151.19±686.52 to 1495.54±812.40 [ng*h/mL]) and $AUC_{0-\infty}$ (from 1229.15±751.04 to 1619.37±955.01 [ng*h/mL]) while $k_{el}$ was decreased and the mean $t_{1/2}$ was prolonged. With regard to 4-hydroxyatomoxetine-O-glucuronide, $C_{max}$ was reduced from 688.76±270.27 to 621.60±248.82 [ng/mL] after coadministration of atomoxetine and duloxetine. **Conclusions**: Duloxetine had an impact on the pharmacokinetics of atomoxetine as it increased the exposure to the latter by ~30%. Although the magnitude of this pharmacokinetic interaction is rather small, a potential clinical relevance cannot be ruled out with certainty without further investigation.

**Keywords**: atomoxetine, 4-hydroxyatomoxetine-O-glucuronide, duloxetine, pharmacokinetic interaction

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## Introduction

Atomoxetine, a selective and potent norepinephrine reuptake inhibitor, is the first nonstimulant agent indicated for the management of attention deficit hyperactivity disorder (ADHD) in pediatric and adult patients [1–3]. With a rapid absorption from the gastrointestinal tract, it reaches peak plasma concentrations ($C_{max}$) within 1-2 hours after oral intake [1,3]. The biotransformation process involves three metabolic pathways: aromatic ring-hydroxylation, benzylic hydroxylation and N-demethylation. The first one is the most important biotransformation step, is mainly mediated by CYP2D6 and leads to formation of 4-hydroxyatomoxetine. The latter is the primary and only active metabolite of atomoxetine and is equipotent to the parent drug as an inhibitor of the norepinephrine transporter. However, it is rapidly inactivated by glucuronidation and eliminated in the urine [3–5]. Given the genetic polymorphism of CYP2D6, the bioavailability of atomoxetine can vary between 63% in individuals considered extensive metabolizers (EMs) and 94% in those characterized as poor metabolizers (PMs); the mean plasma elimination half-life ($t_{1/2}$) ranges between 5.2 hours in EMs and 21.6 hours in PMs [1,3]. Most of the oral dose (80-96%) is eliminated as glucuronidated metabolites *via* urinary excretion while less than 3% is excreted as unchanged drug [3,5].

Duloxetine, a serotonin-norepinephrine reuptake inhibitor, is widely recommended for the treatment of depression and generalized anxiety disorder. Apart from psychiatric conditions, it is also used to treat diabetic neuropathic pain, stress urinary incontinence and fibromyalgia [6,7]. Following oral administration, duloxetine reaches $C_{max}$ in about 6 hours and has a bioavailability that ranges from 32% to 80% [8]. With a $t_{1/2}$ of approximately 10-12 hours, steady-state levels can be achieved within 3 days. This compound is not only a substrate, but also a moderate inhibitor of CYP2D6 [6,8].

Scientific sources report that a depressive disorder is 2 to 4 times more likely to appear for 30 to 60 % of adults diagnosed with ADHD [9]. Based on the recommendations of The Canadian Network for Mood and Anxiety Treatments (CANMAT) task force, pharmacotherapeutic agents for ADHD, including atomoxetine, can be considered as add-ons to antidepressant agents in patients diagnosed with mood disorders and comorbid ADHD [10]. The 2019 European Consensus Statement regarding the diagnosis and treatment of adult ADHD also underlines the fact that combined psychopharmacology may be fre-

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quently needed due to a high rate of psychiatric comorbidity [11]. Duloxetine could be considered a viable option in these circumstances as some preliminary promising results reported that it can improve ADHD symptoms in children, adolescents and adults [12,13].Therefore, as the data supports the hypothesis of a potential concomitant administration of atomoxetine and duloxetine in clinical practice and considering their common metabolic pathway, the objective of this study was to investigate whether the two drugs are involved in a metabolic drug interaction, in healthy subjects.

## Methods

### Participants

The study population comprised Caucasian, healthy, non-smoking men and women (age range: 18-55 years; body mass index (BMI) ≤25 kg/m$^2$). Exclusion criteria included significant medical or medication history that can alter drug response and identification of any abnormal findings during various evaluation tests (clinical examination, electrocardiogram (ECG) and blood tests (hematology - complete blood count; biochemistry - sodium, potassium, calcium, transaminases (aspartate transaminase (AST) and alanine transaminase (ALT)), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), urea, glucose, uric acid, cholesterol and triglycerides, creatinine, total bilirubin and total serum protein levels, immunology and serology tests - screening for pregnancy, human immunodeficiency virus (HIV), syphilis, hepatitis B and C). Those with a history of alcohol or substance abuse and those unable to comply with the study requirements were also considered not eligible. Follow-up visits were performed approximately 30 days after the end of the trial.

### Ethical approval

The clinical trial was carried out in accordance with the requirements of Good Clinical Practice (GCP) and the ethical standards included in the 1964 Declaration of Helsinki and its later amendments. Furthermore, an appropriate ethics committee (Ethics Committee of the University of Medicine and Pharmacy "Iuliu Hatieganu" from Cluj-Napoca, Romania) reviewed and approved the study protocol. Each volunteer provided a written informed consent before any study-related procedures were initiated.

### Study design

The single-site study included 2 periods (Period 1:Reference and Period 2:Test) and used a prospective, open-label and sequential design, without randomization, to determine the effect of multiple-dose duloxetine on the pharmacokinetics of atomoxetine. During Period 1, subjects received a single oral dose of atomoxetine 25 mg (Strattera®, atomoxetine hydrochloride 25 mg, capsules, manufactured by Lilly SA, Madrid, Spain). During Period 2, they were given a combination of duloxetine 30 mg (Cymbalta®, duloxetine hydrochloride 30 mg, delayed-release capsules, manufactured by Lilly SA, Madrid, Spain) and atomoxetine 25 mg, after a pretreatment regimen with duloxetine (*Figure 1*).

More specifically, before the concomitant administration of the two study drugs, a loading dose of duloxetine (60 mg/day) was given to all subjects, for 2 days, in order to speed up the process of reaching steady-state levels and thus ensuring a maximum inhibitory effect. Afterwards, the dose of the enzymatic inhibitor was reduced to 30 mg/day (2 days) to lower the risk of adverse effects. Overall, the chosen dosing regimen took into consideration the need to rapidly achieve steady-state concentrations for duloxetine while minimizing potential safety concerns and the importance of using dosing patterns usually encountered in clinical practice. The medicines were administered in the morning, under fasting conditions and only with water (≥ 150 mL).Volunteers were asked to abstain from consumption of methylxanthine-containing beverages for 2 days prior to the start of the clinical trial and throughout the entire study period. Intake of any other drug except the study medication and oral contraceptives was not permitted during the course of the trial. Alcohol consumption and smoking were also not allowed.

### Blood sample collection and analysis

During both study periods, blood samples (5 ml) were collected on Day 1 (Reference) and Day 6 (Test) into sodium heparin-containing tubes, predose and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, 36 and 48 hours after oral administration of atomoxetine (*Figure 1*). Plasma samples were obtained by centrifugation at 9000 rotations per minute (rpm), for 6 minutes, and were stored at -20$^0$C until their analysis.

High-performance liquid chromatography/tandem mass spectrometry assay (LC-MS) was used to determine the plasma concentrations of atomoxetine and its main metabolite. All LC-MS analyses were performed on an Agilent 1100 system equipped with a binary pump, autosampler and thermostat (Agilent Technologies, Santa Clara, CA, USA) and coupled with a Brucker Ion Trap SL (Brucker Daltonics GmbH, Bremen, Germany). Chromatographic separation was carried out on a Zorbax SB-C18, (Agilent Technologies) column, 100 mm x 3.0 mm i.d, 3.5 µl. The operating conditions included the following: mobile phase (2 mM ammonium formate solution/acetonitrile mixture), flow rate (1 mL/min), gradient program (at start → 11% acetonitrile; after 2 minutes → 41% acetonitrile), column temperature (48°C ). Ionization was achieved by using electrospray in the positive ion mode; the ions monitored were m/z 256 for the parent drug (atomoxetine) and m/z 448 for its main metabolite (4-hydroxyatomoxetine-*O*-glucuronide). Atomoxetine retention time was 4.1 minutes while 2.2 minutes was the value corresponding to 4-hydroxyatomoxetine-*O*-glucuronide. The parameters used to validate the analytical method were linearity, specificity, intra- and inter-day precision, accuracy and analyte

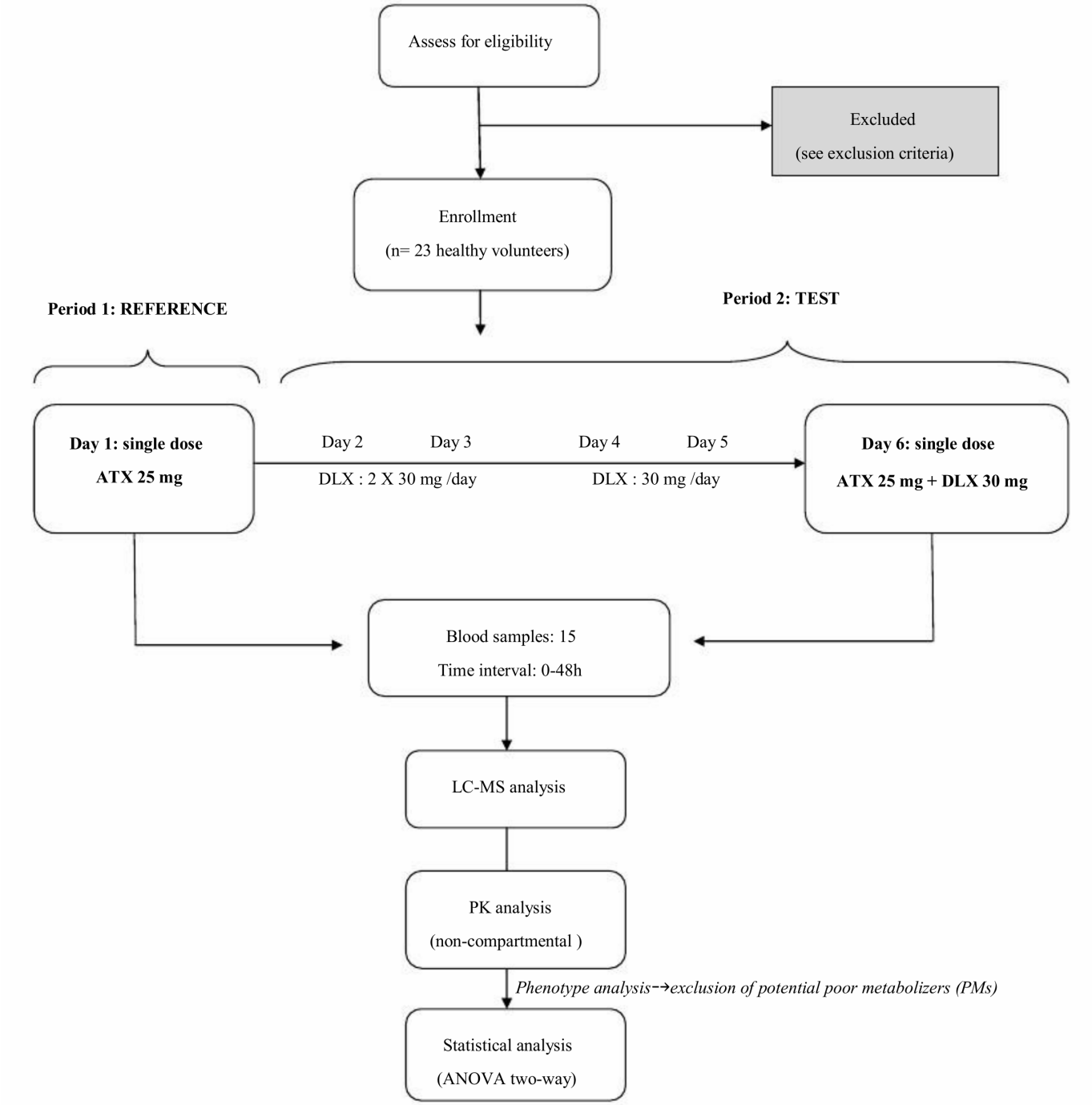


**Fig. 1. Flowchart of the two study periods. Abbreviations: ANOVA - analysis of variance; ATX - atomoxetine; DLX - duloxetine; LC-MS - liquid chromatography–mass spectrometry; PK - pharmacokinetic.**

recovery. The calibration curves were linear over a range of 8-600 ng/mL for both analytes; the correlation coefficients (mean ± standard deviation (SD), n = 5) were as follows: r=0.9951±0.0016 for atomoxetine, r= 0.9982±0.0018 for 4-hydroxyatomoxetine-*O*-glucuronide. The intra- and inter-day precision value was <8.2% for the parent drug and <10.7% for the glucuronidated active metabolite whereas the accuracy yielded a percentage of less than 11.5% and less than 9.3%, respectively. Their average recoveries were in the range of 89-103% for atomoxetine and between 91 and 105% for 4-hydroxyatomoxetine-*O*-glucuronide.

### Pharmacokinetic analysis

The pharmacokinetic analysis was performed using Phoenix WinNonlin® software (Pharsight Co., Mountain View, CA, USA), version 6.3. The non-compartmental method was employed to determine the pharmacokinetic parameters of atomoxetine and 4-hydroxyatomoxetine-*O*-glucuronide, corresponding to both study periods (Reference/Test). The maximum plasma concentration ($C_{max}$, ng/ml) and time to reach $C_{max}$ ($t_{max}$, h) were obtained directly from the plasma concentration-time curves. The elimination half-life ($t_{1/2}$, h) was calculated as $0.693/k_{el}$, where $k_{el}$ ($h^{-1}$), the elimina-

tion rate constant, was the slope of log-linear regression of the terminal phase of the concentration-time curve. The area under the concentration-time curve (AUC) from time 0 to the last quantifiable concentration ($AUC_{0-t}$, ng*h/mL) was obtained by using the linear trapezoidal method. Finally, the AUC extrapolated to infinity ($AUC_{0-\infty}$, ng*h/mL) was estimated as $AUC_{0-t}+C_t/k_{el}$, where $C_t$ represents the last measurable concentration.

### Phenotype analysis

CYP2D6 phenotype status was assessed for each subject by using the $AUC_{0-\infty}$ metabolic ratio (MR_ $AUC_{0-\infty}$: $AUC_{0-\infty}$ _atomoxetine/ $AUC_{0-\infty}$_4-hydroxyatomoxetine-*O*-glucuronide). This calculus was done with the purpose of identifying all subjects characterized as potential PMs and subsequently ensuring their exclusion from the final analysis.

### Statistical analysis

To compute the sample size for the differences between pharmacokinetic parameters, we used G*Power® (Germany), version 3.1.9.4 [14]. The simulations aimed for a power of 90%, with a level of significance of 0.05, for paired t-test, and a two-tailed p-value. We checked for different scenarios with correlation coefficients ranging from 0.01 to 0.99. We started the simulations with data from articles comparing atomoxetine 25 mg with different inhibitors like fluvoxamine [15], bupropion [16], and paroxetine [17]. From these articles, we used the average of the means of the $AUC_{0-\infty}$, the means of the standard deviations and the maximum of the standard deviations for worse scenarios. The majority of the simulations gave sample sizes ranged between 6 and 15, except the worse ones around 26. Thus, we aimed to enroll close to 25 subjects in our study.

Analysis of variance (ANOVA) with 2 sources of variation (subjects and study treatment) was conducted to detect differences between the pharmacokinetic parameters (except $t_{max}$) of atomoxetine and its active metabolite, in the presence and absence of duloxetine. (Test (vs) Reference). A second statistical method, the non-parametric assay known as the Friedman test, was used to compare the mean $t_{max}$ values between study periods. The analyses were performed using Phoenix WinNonlin® software (Pharsight Co., Mountain View, CA, USA), version 6.3. Statistical significance was defined as $p<0.05$.

### Bioequivalence analysis

This methodology was used to obtain preliminary data regarding potential clinical consequences attributed to concomitant atomoxetine and duloxetine intake. Schuirmann's two one-sided test procedure, an equivalence testing approach, was used to calculate the 90% confidence intervals (90% CIs) of the ratio (Test/Reference) for $C_{max}$, $AUC_{0-t}$ and $AUC_{0-\infty}$ (log transformed). In order to fulfill the bioequivalence criteria, the 90% CI values should be included within the acceptance interval of 0.80 -1.25; for $t_{max}$, the range was expressed as untransformed data while the Friedman assay was used to establish significance level. The bioequivalence analysis followed the same protocol for both parent drug and glucuronidated active metabolite and was performed using Phoenix WinNonlin® software (Pharsight Co., Mountain View, CA, USA), version 6.3.

## Results

### Phenotypic assessment

An individual assessment of the MR_ $AUC_{0-\infty}$ showed that the calculated values followed a normal distribution for 22 of the 23 volunteers initially included in the study (data not shown). Subsequently, the 22 subjects were considered to be EMs and were included in the final study sample. On the other hand, 1 subject proved to be an outlier and a potential PM which led to his exclusion from the final data analysis and interpretation.

### Demographic data

The 22 Caucasian EMs included 15 men and 7 women with ages ranging between 20 and 30 years. Mean (±SD) BMI was 24.09±3.09 kg/m$^2$.

### Pharmacokinetic and statistical analysis

The mean plasma concentration-time profiles of atomoxetine [A] and its main metabolite, 4-hydroxyatomoxetine-*O*-glucuronide [B], when administered alone or in combination with the enzymatic inhibitor, duloxetine, are presented in *Figure 2*.

The following tables include the mean pharmacokinetic parameters of atomoxetine (*Table I*) and its glucuronidated active metabolite (*Table II*), for each treatment phase, and the main findings of the statistical tests used for comparison (Test vs Reference).

### Bioequivalence analysis

The 90% CIs for both parent drug and active metabolite and the bioequivalence results are presented in *Table III*.

### Safety evaluation

No clinically significant changes in vital signs, ECG and laboratory parameters were found when the health status of each subject was reassessed after the end of the trial. Special attention was given to the evaluation of the liver function before and after the administration of the study drugs. Thus, the following mean values (±SD) for ALT and AST were recorded: 14.94±9.51 vs 13.30±10.02 UI/l (ALT) and 16.75±5.02 vs 16.53±5.11 UI/l (AST) for female subjects (normal values: 5-33 UI/l (ALT), 5-32 UI/l (AST)), 21.95±9.55 vs 16.20±5.24 UI/l (ALT) and 22.97±12.61 vs 17.86±3.52 UI/l (AST) for male subjects (normal values: 5-41 UI/l (ALT), 5-40 UI/l (AST)).

No serious adverse events were reported and all the volunteers completed the study.

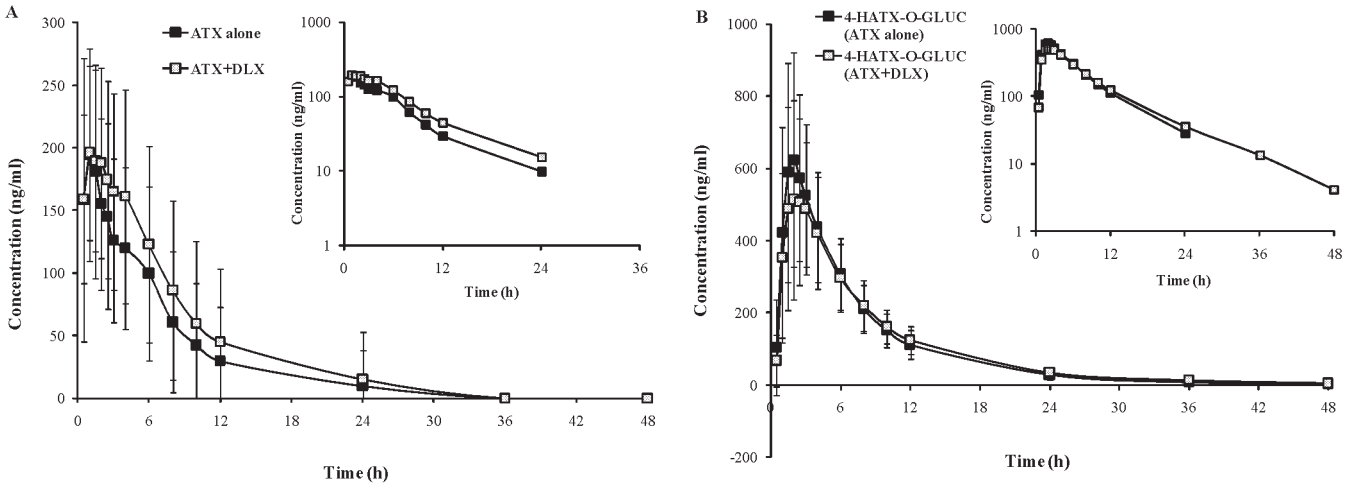


Fig. 2. Mean (±SD) plasma levels of atomoxetine (ATX; [A]) and its glucuronidated active metabolite, 4-hydroxyatomoxetine-O-glucuronide (4-HATX-O-GLUC; [B]), after a single oral dose of ATX 25 mg, before and after a 4-day pretreatment regimen with duloxetine (DLX: 30-60 mg/day), in 22 extensive metabolizers (EMs); Insert: semilogarithmic presentation.

Table I. Pharmacokinetic (PK) parameters for atomoxetine (ATX) after oral administration of 25-mg ATX alone (single dose) and following a 4-day pretreatment drug regimen with duloxetine (DLX) (30-60 mg/day), in 22 extensive metabolizers (EMs) and the statistical test results after interperiod comparison

| PK parameters (mean ± SD) | ATX (Period 1: ATX monotherapy) | ATX (Period 2: ATX+ DLX) | p* value (ANOVAa) |
|---|---|---|---|
| $C_{max}$ (ng/mL) | 221.26±94.93 | 226.07±57.83 | 0.411468, NS |
| $t_{max}$ (h) | 1.30±1.20 | 1.48±1.14 | Friedman, NS |
| $AUC_{0-t}$ (ng*h/mL) | 1151.19±686.52 | 1495.54±812.40 | 0.000325, S |
| $AUC_{0-\infty}$ (ng*h/mL) | 1229.15±751.04 | 1619.37±955.01 | 0.000158, S |
| $k_{el}$ (1/h) | 0.23±0.08 | 0.17±0.09 | 0.004857, S |
| $t_{1/2}$ (h) | 3.57±1.71 | 4.91±2.01 | 0.004857, S |

*Statistically significant (S) at p< 0.05; statistically non-significant (NS); [a]ANOVA except explicitly stated otherwise

Table II. Pharmacokinetic (PK) parameters of 4-hydroxyatomoxetine-O-glucuronide (4-HATX-O-GLUC) after oral administration of 25-mg ATX alone (single dose) and following a 4-day pretreatment drug regimen with duloxetine (DLX) (30-60 mg/day), in 22 extensive metabolizers (EMs) and the statistical test results after interperiod comparison

| PK parameters (mean ± SD) | 4-HATX-O-GLUC (Period 1: ATX monotherapy) | 4-HATX-O-GLUC (Period 2: ATX+ DLX) | p* value (ANOVAa) |
|---|---|---|---|
| $C_{max}$ (ng/mL) | 688.76±270.27 | 621.60±248.82 | 0.013971, S |
| $t_{max}$ (h) | 2.07±0.73 | 2.18±0.80 | Friedman, NS |
| $AUC_{0-t}$ (ng*h/mL) | 4810.93±845.06 | 4842.92±958.21 | 0.931475, NS |
| $AUC_{0-\infty}$ (ng*h/mL) | 4928.55±853.25 | 4958.99±944.73 | 0.895879, NS |
| $k_{el}$ (1/h) | 0.13±0.03 | 0.11±0.02 | 0.000900, S |
| $t_{1/2}$ (h) | 5.71±1.47 | 6.59±1.48 | 0.000900, S |

*Statistically significant(S) at p< 0.05; statistically non-significant (NS); [a]ANOVA except explicitly stated otherwise

Table III. Bioequivalence analysis of the pharmacokinetic (PK) parameters of atomoxetine (ATX) and its glucuronidated active metabolite (4-HATX-O-GLUC), before and after a 4-day pretreatment regimen with duloxetine (30-60 mg/day), in 22 extensive metabolizers (EMs)

| Analyte | PK parameters | 90% CIa | Bioequivalence conclusionb |
|---|---|---|---|
| ATX | $C_{max}$ | 0.93-1.21 | Bio-eq |
| | $AUC_{0-t}$ | 1.21-1.56 | Bio-ineq |
| | $AUC_{0-\infty}$ | 1.22-1.56 | Bio-ineq |
| | $t_{max}$ | Friedman | Bio-ineq |
| 4-HATX-O-GLUC | $C_{max}$ | 0.84-0.96 | Bio-eq |
| | $AUC_{0-t}$ | 0.97-1.02 | Bio-eq |
| | $AUC_{0-\infty}$ | 0.97-1.02 | Bio-eq |
| | $t_{max}$ | Friedman | Bio-eq |

[a]90% CI- 90% confidence intervals; [b]Bio-equivalent (Bio-eq) if 90% CI: 0.8-1.25; Bio-ineq: Bio-inequivalent

## Discussion

ADHD is one of the most common childhood neurodevelopmental disorders, characterized by inattention, impulsivity and motor hyperactivity [18]. Nonetheless, the perception that this illness is restricted to children and adolescents is not accurate, as more than 50 % of those diagnosed with ADHD can experience part of the symptoms in adulthood [19,20]. A meta-analysis conducted by Willcutt *et al.* reported a prevalence of 5.9 -7.1 % for ADHD in children and adolescents [21] whereas for the adult population, The World Mental Health Survey Initiative established a prevalence rate ranging from 1.2 % to 7.3 % in a study that included ten countries across Americas, Europe and Middle East [22]. As atomoxetine is one of the main agents used to treat ADHD [1] and data regarding its pharmacokinetic

interactions are limited, the present study considered providing new information regarding its safety profile by investigating a potential drug interaction with duloxetine.

Since CYP2D6 is highly polymorphic and CYP2D6 inhibitors have little or no impact on atomoxetine pharmacokinetics in PMs [3], all data related to the subject identified as potential PM was removed from the final analysis in order to avoid any interference with the study outcomes. Besides the CYP2D6 PM status, two other potential confounding factors should be addressed. First, the use of hormonal steroid contraceptives can be problematic as the scientific literature provides evidence that compounds such as progesterone, pregnanolone, pregnenolone, 17β-estradiol, and 17β-hydroxyprogesterone are substrates and inhibitors of CYP2D6 [23]. However, even though the use of oral contraceptives was not considered exclusion criteria, none of the female subjects reported using this type of medication which disproves the hypothesis regarding a possible interference with the study results. Second, atomoxetine exposure can be increased when hepatic impairment is present [5] and, in some cases, duloxetine use was associated with hepatic injury [24]. However, in this study, no significant increases of liver transaminases were reported after treatment with the antidepressant and as a result, duloxetine-induced hepatotoxicity was also excluded as a potential interfering factor.

In the present research, the mean plasma concentration-time profile illustrated in *Figure 2 (A)* showed that a 4-day pretreatment with duloxetine produced a moderate increase in atomoxetine plasma concentrations. Contrarily, the mean plasma concentrations of the glucuronidated active metabolite suffered a slight decrease during the Test period (*Figure 2 (B)*) due to enzymatic inhibition, as the process slowed down the biotransformation of the substrate and the production of metabolite.

The pharmacokinetic analysis revealed that most of the calculated parameters for atomoxetine showed statistically significant changes between study periods (*Table I*). For example, the enzymatic inhibitor caused a 1.3-fold (~ 30%) increase in both $AUC_{0-t}$ and $AUC_{0-\infty}$ for atomoxetine. The increased exposure can be interpreted as an indicator for the existence of a metabolic interaction between atomoxetine (CYP2D6 substrate) and duloxetine (CYP2D6 enzymatic inhibitor). Moreover, compared to Period 1, when atomoxetine was administered alone, during Period 2, after duloxetine pretreatment, a 26% decrease was reported for $k_{el}$ value while atomoxetine $t_{1/2}$ was prolonged by 37.5%. This suggests that, in this case, the clearance of the parent drug was reduced under the influence of the enzymatic inhibitor. As for $C_{max}$ and $t_{max}$, no statistically significant differences were observed between study periods. In addition, the pharmacokinetic profile of the glucuronidated active metabolite (4-hydroxyatomoxetine-*O*-glucuronide) confirmed the drug-drug interaction. Duloxetine pretreatment significantly influenced three pharmacokinetic parameters of this compound as it decreased $C_{max}$ and $k_{el}$ by 9.7% and 15.3%, and increased the mean $t_{1/2}$ value by ~ 15% (*Table II*).

Up until now, a relatively small number of studies provided information about the pharmacokinetic interactions of atomoxetine. Previous trials that evaluated the impact of other CYP2D6 inhibitors on atomoxetine pharmacokinetics concluded that paroxetine [25], bupropion [16] and fluvoxamine [15] increased the exposure to this agent by approximately 6.5-, 5.1- and 1.3-fold, respectively. In comparison with these antidepressants, duloxetine had only a modest impact on atomoxetine pharmacokinetics, comparable to fluvoxamine but much more reduced than paroxetine.

According to the bioequivalence analysis, the 90% CIs of $t_{max}$, $AUC_{0-t}$ and $AUC_{0-\infty}$ corresponding to atomoxetine were not in the acceptable limit range (*Table III*), which could indicate a potential clinical relevance in this case. Even though bioequivalence was established for the rest of the pharmacokinetic parameters and the results revealed only a small magnitude for this pharmacokinetic interaction, any conclusion with regard to potential clinical outcomes cannot be drawn without additional investigations. Until then, caution is required whenever atomoxetine and duloxetine are concomitantly administered in clinical practice as the consequences of this pharmacokinetic interaction are not precisely known. In trials that included adult patients, the most frequently reported adverse events of atomoxetine were nausea, dry mouth and decreased appetite. Similar side effects (headache, abdominal pain and decreased appetite) were noted for children [26]. Several studies found slight increases in blood pressure and heart rate during treatment with atomoxetine which suggests that monitoring of cardiovascular parameters should be taken into consideration for safety purposes [27,28]. In a case report published in 2011, the addition of fluoxetine to the medication regimen of a 26 years patient who had been receiving atomoxetine for the past 6 years, led to an increased exposure to the ADHD agent, which caused the patient to experience cardiovascular side effects such as syncope, orthostatic hypotension and tachycardia [29]. Furthermore, whenever atomoxetine is coadministered with duloxetine, a potential pharmacodynamic interaction can also be present as the latter might cause additive increases in blood pressure [11].

### Limitations

The absence of genotyping data that could have confirmed the phenotype analysis results can be considered as an important limitation of the present research. In addition, we acknowledge the fact that this study only focused on pharmacokinetic aspects and did not provide any useful information regarding the clinical relevance of this pharmacokinetic interaction.

## Conclusion

Exposure to atomoxetine was increased after pretreatment with duloxetine. Thus, it can be concluded that the antide-

pressant has an impact on atomoxetine pharmacokinetics, but supplementary studies, preferably with a multiple-dose atomoxetine regimen, are needed in order to provide information with respect to any potential clinical consequences. Although the clinical relevance is not yet known, this research offers some insight that could be helpful to clinicians in the process of treatment selection in patients with ADHD and comorbid psychiatric disorders.

## Conflicts of interest

None to declare.

## Authors' contribution

Ioana Todor (Data curation; Writing – original draft)
Adina Popa (Methodology; Writing – review & editing)
Dana Muntean (Data curation)
Maria Neag (Investigation; Supervision)
Ana-Maria Gheldiu (Data curation; Writing – review & editing)
Corina Briciu (Data curation; Formal analysis; Writing – original draft)
Daniel Leucuta (Formal analysis; Methodology; Writing – review & editing)
Laurian Vlase (Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing – review & editing)

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CASE REPORT

# Genetic Investigation and Clinical Aspects in a Romanian Treacher Collins Syndrome Family – A Case Report

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**Introduction**: In approximately 96% of probands, the diagnosis of Treacher Collins Syndrome (TCS) is confirmed by molecular genetic tests. These tests can detect heterozygous mutation of TCOF1 gene (coding treacle protein) and variants of POLR1D gene (coding RNA polymerase I subunit D) with autosomal dominant inheritance, or biallelic variants of POLR1C gene (coding RNA polymerase I subunit C) and POLR1D with autosomal recessive inheritance. **Case presentation**: We present a neonate proband with family history of clinical features suggestive for TCS. Our patient was investigated for copy number changes (CNCs) of TCOF1 gene using SALSA MLPA P310-B3 TCOF1 probemix to perform Multiplex Ligation-dependent Probe Amplification (MLPA), the results being normal. Dysmorphic features revealed "bird-like" face with trigonocephaly, craniosynostosis, hypoplastic supraorbital rims, underdeveloped zygomas, mandibular hypoplasia and retrognathia (mandibulofacial dysostosis). Other clinical features, like abnormal position and structure of the external ears (microtia, with a bilateral low-set ears, crumpled and malformed pinnae and aural atresia), were also observed. **Conclusion**: Taking into account our results, and also data found in literature, we consider that all TCS cases, but in particular patients with specific TCS features and without CNCs, require additional investigations using sequencing techniques.

**Keywords**: TCOF1 gene, Treacher Collins syndrome, MLPA technique, neonate

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## Introduction

In 1846, Thomson described for the first time a new syndrome, followed by the work of Berry (1889), Treacher Collins (1900) and completed by Franceschetti and Klein in 1949 [1]. Treacher Collins syndrome (TCS, OMIM 154500), named also Treacher Collins-Franceschetti syndrome or mandibulofacial dysostosis (MFD) arises through a reduction mechanism in the number of cranial neural crest cells given by a haploinsufficiency of the treacle protein. The cells subsequently migrating to the first and second pharyngeal arches [2]. This condition develops between the 20th day and the 12th week of intrauterine life and it is an autosomal dominant disorder of craniofacial development occurring in one in 50,000 live births [3, 4]. Even among members of the same family, signs, symptoms and severity of this disorder can vary greatly from one person to another, differing from almost imperceptible to severe.

In approximately 96% of probands, the diagnosis of TCS is confirmed by molecular genetic tests. These tests can detect heterozygous mutation of TCOF1 gene (coding treacle protein) and variants of POLR1D (coding RNA polymerase I subunit D) with autosomal dominant inheritance, or biallelic variants of POLR1C (coding RNA polymerase I subunit C) and POLR1D with autosomal recessive inheritance. In the remaining 4% of probands, when molecular genetic testing does not detect allelic variants in either of the known genes, the diagnosis of this condition is established based on clinical findings only [5].

Thus far, *TCOF1* gene is known to be implicated in more than 90% of cases with TCS and approximately 130 distinct mutations including insertion, splicing and nonsense mutations with a distribution along 26 exons located on 5q33.1 locus, have been described in the literature [6, 7]. Moreover, less than 40% of patients have a family history of TCS, while de novo appearance of this condition arises in 60% of the cases [8, 9]. As stated by Mendelian laws of genetics, an affected parent may transmit the pathogenic variant to the offsprings with a risk of 50%, which highlights the importance of genetic counseling in the case of affected individuals and their families [3]. The present report illustrates the clinical findings and molecular analysis of TCS on a neonate with a family history of this condition, without molecular testing being performed on the family members.

## Case presentation

We describe the case of a late preterm Caucasian male newborn, small for gestational age who was the product of a primigravid mother and non-consanguineous parents. The pedigree analysis showed a positive family history for congenital bilateral hearing impairment in the maternal lineage, in the first and second degree (Figure 1). The newborn was delivered by Cesarean section (C-section) at only 36 weeks of gestation because of maternal pregnancy-induced hypertension (PIH) and severe preeclampsia. The amniotic

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fluid was clear and Apgar scores were 8 at 1 minute and 9 at 5 minutes. Birth weight was 1850 grams (<3$^{th}$ percentile) with a 46 cm length (> 10$^{th}$ centile), 30 cm head circumference (<10$^{th}$ centile) and appropriate postpartum adaptation.

Clinical evaluation of the newborn revealed a "bird-like" face with trigonocephaly, craniosynostosis, hypoplastic supraorbital rims, underdeveloped zygomas, mandibular hypoplasia and retrognathia (mandibulofacial dysostosis). The newborn also presented antimongoloid slant of palpebral fissures, microphthalmia and coloboma, without stenosis or choanal atresia. Other clinical features, like abnormal position and structure of the external ears (microtia with a bilateral low-set ears, crumpled and malformed pinnae and aural atresia), were also observed.

Cranial ultrasonography revealed no additional abnormalities and described structures being in relationship with gestational age. 2D-echocardiography showed ostium secundum atrial septal defect < 3 mm with a septal aneurysm and left-to-right cardiac shunt, but normal cardiac chambers. Ophthalmologic examination highlighted clear and well-defined borders of optic disc with immature retinal vascularization of zone III and a normal macula. Furthermore, a bilateral iris and chorioretinal coloboma on the inferonasal quadrant was observed. Pediatric surgical evaluation confirmed our suspicion of hypospadias, based on the physical exam. In evolution, abdominal ultrasound performed at 4 weeks of life revealed mild bilateral hydronephrosis. Over time, hydronephrosis showed a slight improvement of the grade, at the time of follow-up requiring only ultrasound monitoring. Native computer tomography (CT) of the head was performed, describing the inner ear, as well as the internal auditory canal (IAC) within normal limits. It also described, the presence of tympanic cavity, but with a poor visualization of the bilateral auditory ossicular system and narrowed external auditory canals (EAC). The patient was referred to the medical genetics department where the mother was observed to present similar peculiar facial aspects, but with lower clinical phenotype expression severity. In the upcoming weeks the newborn was feeding well without functional respiratory disorder. He had an adequate evolution and was discharged after the first month of life with medical recommendations.

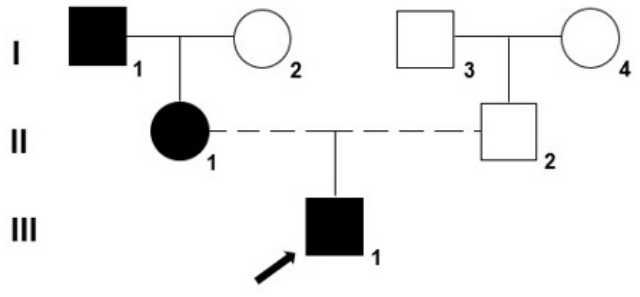


Fig. 1. Pedigree chart of our patient with autosomal dominant inheritance of TCS in the family

For genetic analysis, genomic DNA was extracted from venous peripheral blood of the newborn and also from the mother, using PureLink Genomic DNA kit (ThermoFisher, Massachusetts, USA). The study was approved by the Ethics Committee of the University of Medicine and Pharmacy Tîrgu Mureş, Romania (No. 47 from February 23, 2018). Informed written consent for testing and publication was obtained from the parents.

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was reported as a useful, fast and low-priced method to evidence copy number changes (CNCs) in several genes [10, 11]. On the other hand, several pathogenic CNCs are known to be implicated in TCS. As a result, we performed MLPA analysis using SALSA MLPA P310-B3 *TCOF1* probemix from MRC-Holland (Amsterdam, Netherlands). According to the manufacturer description, the probemix contains one probe for every exon of the *TCOF1* gene, excepting exons 8, 19 and 20. Furthermore, a probe for intron 6 and another one for intron 16 were included in this probemix along with 10 reference probes for different locations on the autosomal chromosomes. Denaturation of DNA, reaction of hybridization and ligation, followed by polymerase chain reaction (PCR) and capillary electrophoresis for fragment separation were performed as previously described, according to the manufacturer instructions [12].

We performed fragment analysis by capillary electrophoresis with 50 cm array and POP-7 polymer, using Applied Biosystems 3500 Genetic Analyzer. Final results were obtained using the Coffalyser.Net software taking into account the normal ratio, for the region of interest, between 0.7 to 1.3.

Prominent TCS clinical findings were notice, in the proband. In addition, congenital bilateral hearing impairment and craniofacial abnormalities, but with lower clinical severity were discovered in his mother as well. Similar phenotype was also noticed in his grandfather who has been clinically diagnosed with TCS.

In both cases, neonate and mother, the results of MLPA analysis failed to detect the presence of CNCs for *TCOF1* gene, all probes included in the kit being in normal ranges (0.7-1.3).

## Discussion

The present case report was designed to illustrate the clinical findings and MLPA analysis of TCS in a neonate proband with a family history of this condition, without molecular testing being performed on the family members.

It is known that more than 60% of TCS cases have no previous family history of this condition and appear as de novo mutations [8, 9]. Discordance between genotype and fenotype has been reported in several studies, although there is no clear mechanism of occurrence [13, 14]. Despite the fact that the penetration of genetic mutations with regard to TCS is considered to be high, the diagnosis of this syndrome may be overlooked due to the phenotypic

variability of clinical manifestations. However, severe cases that can develop respiratory disturbances leading to perinatal mortality have been described [15]. These features highlight the multifactorial nature of this process, with the implication of genetic factors, environmental factors and stochastic events. Taking into account the severity score introduced by Teber et al., based on clinical features, our patient is severely affected scoring 16 out of 20 points [13].

In order to confirm diagnosis and establish the etiology, genetic molecular tests are required to detect possible mutations in the most commonly reported genes involved, such as *TCOF1*, *POLR1C* and *POLR1D* genes. Localized on 5q32-q33.1, the *TCOF1* gene was found to be implicated in the development of approximately 90-95% of TCS cases.

In a similar study conducted by Beygo J et al [16] on a cohort of 112 patients investigated with the same technique, MLPA analysis did not confirm the presence of CNCs in the *TCOF1*, *POLR1C* and *POLR1D* genes, and revealed only one aberrant signal intensity in exon 3 for one patient. As long as there were not at least two consecutive aberrant signals, based only on MLPA results, it could not be considered a deletion. However, this one aberrant signal suggested the need of targeted additional molecular investigations such as long-range PCR and sequencing technique. Therefore, Beygo J et al were able to identify deletion of the complete sequence of exon 3 with the interest of introns 2 and 3 [16]. For the rest of the patients, where all MLPA probes were in normal ranges, whole gene sequencing was needed. In accordance with our study, Beygo J et al highlights the need for further analysis for point mutations.

A study that focused on phenotypic characteristics and molecular diagnosis using Sanger sequencing of *TCOF1* gene, included a single TCS family. In this research, Han et al [17], identified a heterozygous mutation in exon 3 of the *TCOF1* gene, namely a splice-site c.165-1G> A mutation. The same pathogenic variant was identified in the proband's mother, but not in other relatives, suggesting that a de novo c.165-1G>A mutation may be implicated in the pathogenesis of TCS in this family [17].

A novel 1-bp duplication and a 4-bp deletion identified in the *TCOF1* gene were reported by Caluseriu et al, as further possible pathogenic mechanisms for TCS [18]. Moreover, in the study performed by Vincent et al [19] on 146 subjects with TCS, 63% of patients presented a mutation in the *TCOF1* gene. In 6% of cases, they identified a pathogenic variant of the *POLR1D*, but not of the *POLR1C* gene. In addition, in the same study, among patients with clinical features like microcephaly or intellectual disability, they identified one case with a 5q32 deletion. The deleted sequence included calcium/calmodulin dependent protein kinase II alpha (*CAMK2A)* and *TCOF1* genes. They also identified 4 cases carrying a mutation in Elongation Factor Tu GTP Binding Domain Containing 2 *(EFTUD2)* gene [19]. It has been reported that mutations in the *TCOF1* gene might be responsible for clinical aspects of TCS, as well as mutations in the *CAMK2A* gene for intellectual disability seen in this condition [20]. Genetic analysis of the beforementioned genes was performed by Vincent et al. using the same method as in our present study, and subsequently applying Sanger sequencing method and array-comparative genomic hybridization.

Other two studies [21, 14] including five patients with minimal diagnostic criteria (a newborn and four cases with Turkish origin) and presenting similar clinical features as our patient, described a pathogenic heterozygous deletion in exon 7, c.1021_1022delAG of *TCOF1* gene, using Sanger sequencing [21, 14].

Given the variable expressivity of the clinical symptoms of this syndrome, identifying the causative factors by using a targeted molecular pathway is important for genetic counseling, and treatment strategies of the patient. In our case, the diagnosis of TCS was established based on clinical features of the proband, of his mother and grandfather. Due to the fact that there are several challenges and limitations of molecular analysis in developing countries such as Romania, especially regarding costs, we were unable to sequence the abovementioned genes. Another limitation of our study is the lack of a sample from the grandfather, and the fact that we were unable to investigate the *POLR1C* or *POLR1D* genes (although they can account for up to 5% of TCS cases).

**In conclusion**, taking into account our results, and also data found in literature, we consider that all TCS cases, but in particular patients with specific TCS features and without CNCs, require additional investigations using sequencing techniques.

## Conflict of interest

None to declare.

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