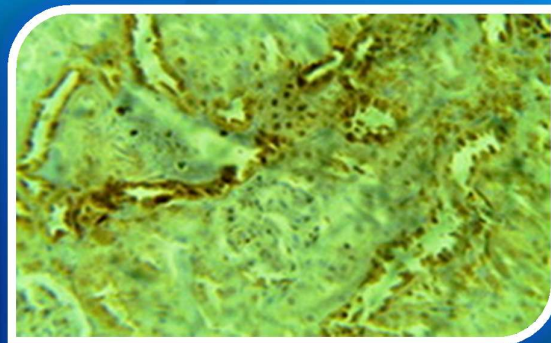


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# EXPERIMENTAL BIOMEDICAL RESEARCH



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## High environmental temperature potentiated marker of oxidative cellular damage and renal expression of p38 MAPK in male rats fed a high salt diet

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### ABSTRACT

**Aim:** Oxidative stress, heat shock protein (HSP70) and p38 mitogen-activated protein kinase (MAPK) are important functional cellular signals involved in the pathophysiology of cardiovascular diseases. This study investigated the effect of high environmental temperature (HET) and high salt diet (HSD) respectively and together on systemic oxidative stress, nitric oxide (NO), HSP70 and renal p38 MAPK.

**Methods:** Thirty-two male Sprague-Dawley rats were divided into four groups with eight rats in each group: Control rats (C) fed a normal diet; salt-loaded rats (S) fed a HSD (8%NaCl); normal diet rats (H) exposed to HET ( $38.5 \pm 0.5^\circ\text{C}$  4h daily for 8weeks); and salt-loaded rats (SH), fed a HSD and exposed to HET. Circulatory oxidative stress parameters (SOD: superoxide dismutase; GSH: glutathione; CAT: catalase; MDA: malondialdehyde), HSP70 and renal p38 MAPK were determined by colorimetric, enzyme-linked immunosorbent assay (ELISA) methods and immunohistochemistry (IHC) techniques respectively.

**Results:** Plasma GSH concentration and CAT activity decreased significantly, with significant increase in MDA concentration in all the rat groups compared to control. However, MDA in SH rats was significantly higher than in either S or H rats. Circulatory HSP70 and NO were significantly raised in S and H rats but unchanged in SH rats compared to control. Conversely, renal expression of p38 MAPK was significantly increased in H and SH rats compared to control, but SH rats had significantly higher level than either S or H rats. SH rats also had weight gain slowing compared to control.

**Conclusion:** Our findings indicate that prolonged exposure to HET and HSD intakes synergistically increased renal p38 MAPK and circulatory product of oxidative cellular damage without alteration in circulatory HSP70 and NO.

**Keywords:** HSP70, p38 MAPK, high environmental temperature, high salt diet, oxidative stress.

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## Introduction

Exposure to high environmental temperature is on the rise worldwide due to global warming [1]. Dietary high salt intake is also rising (>5 grams per day) in many countries around the globe and was reported to be responsible for 3 million deaths from cardiovascular related causes [2]. High environmental temperature has also been suggested as a cardiovascular risk factor [3]. Sadly, cardiovascular disease (CVD) is identified as the leading cause of death and disability worldwide [4]. Our previous work demonstrated that high environmental temperature interacted with high salt diet in experimental rats to exacerbate salt-induced increase in blood pressure and myocardial workload [5]. Oxidative stress has been identified as the main cause of increased mortality in cardiovascular disease [6], with p38 mitogen-activated protein kinase (MAPK) and heat shock protein (HSP) playing an important regulatory roles.

HSP is induced by a wide variety of stimuli including pressure or volume overload, thermal stress, salt-induced stress [7]. HSPs exhibit cytoprotective effects on different organs, and these may include the correction of folding of regulatory proteins, degradation of abnormal protein, protection of cytoskeleton as well as inhibition of apoptosis [8]. HSP70 also enhances nitric oxide production [9]. So far, the presence of HSP70 has been demonstrated in several experimental models of hypertension including salt sensitive hypertension [7].

p38 MAPK is an example of intracellular protein kinase involved in cellular signaling and activated by numerous extracellular stimuli [10]. When activated, p38 MAPK is reported to cause DNA damage and cell death [11]. Its activation has also been shown to upregulate specific inflammatory cytokines in several biological contexts including kidney damage

[12], myocardial injury and spontaneous hypertension in stroke prone rats [13]. This current study is aimed at investigating the plausible role of oxidative stress, HSP70 and alpha p38, otherwise referred to as p38 MAPK, in the earlier reported synergistic interaction between high environmental temperature and high salt diet on cardiovascular function [5].

## Materials and Methods

### *Experimental animals*

The study protocol was approved by the Ethics committee of College of Medicine of the University of Lagos (CMUL/HREC/11/18/471) and was performed at the Department of Physiology Research Lab of the College of Medicine, University of Lagos. Animal care and handlings were done according to the National Research Council (US) Committee for the Care and Use of Laboratory [14]. Thirty-two male Sprague-Dawley rats weighing between 95 and 110g were used for the study. The rats were maintained on a 12h dark/light cycle at  $25 \pm 0.5^\circ\text{C}$  room temperature in the animal house and were allowed access to standard rat chow and clean tap water ad libitum throughout the study. The rats were randomly divided into one of the following experimental groups with 8 rats per group.

Group 1: Control rats (C), were fed with normal diet containing 0.3% of NaCl and exposed to room temperature of  $25 \pm 0.5^\circ\text{C}$  throughout the 8 weeks of the experiment.

Group 2: Salt-loaded rats (S), were fed with high salt diet containing 8%NaCl and exposed to room temperature of  $25 \pm 0.5^\circ\text{C}$  throughout the 8 weeks of the experiment.

Group 3: Heat-exposed rats (H), were fed on normal diet but exposed to high environmental temperature at  $38.5 \pm 0.5^\circ\text{C}$  (relative humidity between 65 and 75%) 4 hours daily for 8 weeks.

Group 4: Salt-loaded + Heat-exposed rats (SH), were fed with high salt diet (containing 8%NaCl) and exposed to high environmental temperature at  $38.5\pm 0.5^{\circ}\text{C}$  (relative humidity between 65 and 75%) 4 hours daily for 8 weeks.

### ***Experimental protocol***

Salt-loaded rats were fed with high salt diet as described by Sofola et al., [15] for 8 weeks. Heat-exposed rats were acclimatized to HET for one week starting from  $30^{\circ}\text{C}$  to  $35^{\circ}\text{C}$  with a daily temperature increase of  $1^{\circ}\text{C}$ . Thereafter, the animals were exposed to HET using the method described by Barney & Kuhrt [16], but with a slight modification of the temperature to a higher level of  $38.5\pm 0.5^{\circ}\text{C}$  and a relative humidity between 65 and 75%. The consideration was due to the relatively high room temperature of our environment located in the South West Nigeria of Sub-Saharan Africa in the tropics. Heat exposure took place for 4 hours daily for 6 (days/week) for 8 weeks in environmental chambers from 9am to 1pm. Environmental temperature was monitored with environmental thermometer (HTC-2 OEM, Zhejiang, China).

### ***Determination of weekly body weight gain***

Percentage body weight gain was determined on weekly basis in all the experimental rats ( $n=8$ ) with digital weighing scale using the following formula:  $\frac{W_c - W_p}{W_p} * 100$ ;  $W_c$ , current weight;  $W_p$ , previous weight.

### ***Animal sacrifice***

The rats were anaesthetized on the 8-week with ketamine (75mg/Kg) intraperitoneally, blood was collected via cardiac puncture into heparinized bottles and centrifuged at 3000rpm for 15 minutes to extract plasma samples, which were stored at  $-25^{\circ}\text{C}$  until further analysis for super oxide dismutase (SOD) and catalase

(CAT) activities, reduced glutathione (GSH), malondialdehyde (MDA) and nitric oxide (NO) concentrations. The rats were perfused through the ascending aorta with 40 ml of 0.01 M phosphate buffered saline (PBS, pH 7.4) containing Heparin (5 IU/mL) at room temperature. This was followed by 400 mL of 4% paraformaldehyde (PFA) in phosphate buffer (PB) 0.1 M sodium (4% PFA, pH 7.4) at  $4^{\circ}\text{C}$ . The kidneys were carefully isolated and removed immediately after perfusion, post-fixed in 4% PFA for 2 hours at  $4^{\circ}\text{C}$  and finally stored at room temperature in 10% formal saline until p38 MAPK study using immunohistochemistry (IHC) technique.

### ***Super oxide dismutase activity***

Super oxide dismutase activity (SOD) activity was determined by the method of Misra and Fridovich [17] based on the principle that superoxide anion ( $\bullet\text{O}_2^-$ ) causes the oxidation of epinephrine to adrenochrome, a transition that is inhibited by SOD (enzymes) in the biological sample. Briefly, 14.3g of  $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$  (Sigma Chemicals Ltd, USA) and 4.2g of  $\text{NaHCO}_3$  (Sigma Chemicals Ltd, USA) were dissolved in distilled water and made up to 1000ml mark in a liter standard flask. Then the solution was adjusted to pH 10.2. Next, 0.0013g of epinephrine (molecular weight of 182.21g/mol) (Sigma Chemicals Ltd, USA) was dissolved in 250ml of distilled water. Thereafter, 0.1ml of plasma was diluted in 0.9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted plasma sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate, and the reaction was started by adding 0.3ml of freshly prepared 0.3mM epinephrine to the mixture which was quickly mixed by inversion. The increase in absorbance at 480nm was monitored every 30seconds for 150 seconds in

spectrophotometers (T70UV/VIS, UK). 1 unit of SOD activity was regarded as the amount of SOD required to cause 50% inhibition of the oxidation of epinephrine to adrenochrome during 1 minute.

#### **Catalase activity**

Catalase (CAT) activity was evaluated colorimetrically according to the method of Sinha [18] by determining the rate of consumption of H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) at absorbance measured with a spectrophotometer at 570nm. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H<sub>2</sub>O<sub>2</sub> with the formation of perchromic. Briefly, different amounts of H<sub>2</sub>O<sub>2</sub> ranging from 20 to 160 mmoles were taken in small test tubes and 2ml of dichromate/acetic acid was added to each. This immediately produces an unstable blue precipitate of perchromic acid. Following the heating of the solution for 10mins in boiling water the color of the solution changed to stable green due to the formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3ml with distilled water and the absorbance was measured with a spectrophotometer at 570nm. Next, 0.1ml of plasma was mixed with 4.9ml of distilled water to give a 1 in 5 dilution of the sample. 1ml of the diluted plasma preparation (test sample) was then rapidly mixed with the reaction mixture by gentle spinning motion at room temperature. Thereafter, 1ml of the reaction mixture was withdrawn and blown into a test tube containing 2ml of dichromate/acetic acid reagent at 60 seconds intervals for 3minutes. The H<sub>2</sub>O<sub>2</sub> contents of the withdrawn sample were determined. The decomposition of H<sub>2</sub>O<sub>2</sub> by catalase was determined by using the equation for a first – order reaction.  $K = 1/t \log S_0/S$ . Where, S<sub>0</sub> is the initial concentration of

H<sub>2</sub>O<sub>2</sub>. S is the concentration of peroxide at t min (60 seconds interval). T is time – interval (1minute).

#### **Reduced glutathione**

Reduced glutathione (GSH) was determined by the method described by Habing et al [19]. Briefly, 0.2ml of plasma sample was mixed with 1.8ml of distilled water to give 1 in 10 dilutions. About 3ml of precipitating reagent (4% sulphosalicylic acid) was added to the diluted plasma sample and then allowed to stand for 10minutes for precipitate to occur. 0.5ml of supernatant was withdrawn and added to 4ml of phosphate buffer followed by 0.5ml of Ellman's reagent. The blank was prepared with 4ml of 0.1M phosphate buffer pH 7.4, 1ml of diluted precipitating solution and 0.5ml of Ellman's reagent (DTNB). The absorbance was read within 20minutes of color development at 412nm against blank using spectrophotometer. Reduced glutathione concentration was proportional to the absorbance at 412 nm.

#### **Malondialdehyde**

Malondialdehyde (MDA) resulting from lipid peroxidation of biological membrane was estimated with the method described by Mihara and Uchyama (1978)[20]. This is based on the formation of pink colour complex when MDA interact with thiobarbituric acid (TBA) at 535nm. Briefly, Trichloroacetic acid (TCA; 15%), hydrochloric acid (HCL: 0.24N) and thiobarbituric acid (TBA; 0.37%) were prepared and mixed in ratio 1:1:1. Thereafter, 1ml of filtrate from centrifuged plasma was added into the 2ml of the acidic solution. The absorbance was read at 535nm in spectrophotometers.

#### **Plasma nitric oxide measurement**

Total plasma concentration of nitrites (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) were used as an indicator of

plasma nitric oxide (NO). Nitrites ( $\text{NO}_2^-$ ) using the Griess reaction [21]. Nitrate and nitrite were measured using the cadmium reduction and diazotization techniques respectively. Concentrations of nitrate and nitrite were determined calorimetrically by reading absorbance with UV-Spectrophotometer, (CAMSPEC 309, and USA) at 500 nm and 507 nm respectively. Briefly, 1ml each of plasma was measured in a volumetric flask with 6 ml of deionized water. In the mixture, 1ml of 5% mercuric chloride was added, made up to 10 ml and filtered into a 25ml standard volumetric flask. The solution was made up to mark with deionized water. A standard preparation and a blank control were also prepared using the same method adopted in preparing the samples except that no plasma was added. 10 ml of each solution were measured in 20 ml test tubes before adding nitrate and nitrite powder pillow reagents (HACH Company, USA) respectively in each. This was then allowed to stand for 20 minutes for a color development and the readings were taken respectively.

#### ***Heat Shock Protein 70 (HSP70)***

Plasma HSP70 concentrations was determined using rat HSP70 elisa kit (MyBiosource, San Diego, USA) according to the manufacturer instruction. Briefly, 0.1ml of plasma sample and 0.1 ml of graded concentration of standard preparation respectively were added into micro wells pre-coated with HSP70 antibody and incubated for 90minutes at 37°C, followed by the addition of 0.1ml of Biotin-label antibody and incubation for 60 minutes at 37°C. The micro wells were washed with buffer water thrice. 0.1ml of SABC solution each was added into each of the well and incubated for 30minutes at 37°C followed by washes five times. Then, TMB substrate (90 $\mu\text{L}$ ) was added into the wells in dark within 15 minutes. A stop

solution (50 $\mu\text{L}$ ) was then added into each of well to stop the reaction. The absorbance was read at 450nm in microplate immediately and used to determine the concentration of HSP70.

#### ***Determination of renal expression of alpha p38 MAPK by immunohistochemistry techniques***

Renal expression of p38 MAPK was determined using Rat IHC-P kit (Cell Signaling Technology, USA) according to the manufacturer instruction briefly described below. ***Kidney section and deparaffinization:*** Kidneys were processed into blocks with liquid paraffin. The blocks were sectioned with microtomes and placed on charge slides. This was followed by the deparaffinization and hydration of the kidney sections by incubating the sections in three washes of xylene for 5 min each, followed by incubation in two washes of 100% ethanol for 10min each and another two washes in 95% ethanol for 10 mins each. Thereafter sections were washed twice in dH<sub>2</sub>O for 5 min each. ***Antigen unmasking:*** Slides were heated in a microwave submersed in 1X citrate unmasking solution until boiling is initiated, followed with 10 min at sub-boiling temperature (95-98°C). The slides were then cooled on bench top for 30 min. ***Staining:*** Sections were washed in dH<sub>2</sub>O three times for 5 min each. The sections were then incubated in 3% hydrogen peroxide for 10 min. This was followed by wash in dH<sub>2</sub>O two times for 5 min each, followed by a wash in buffer for 5 min each. Next, the sections were blocked with 100-400 $\mu\text{L}$  of blocking solution (normal goat serum blocking solution) for 1 hr at room temperature. The blocking solution was removed from the sections and 100-400 $\mu\text{L}$  of primary antibody diluted in Signal stain Antibody Diluent was added to each section, followed by an incubation overnight at 4°C. The primary

antibody solution was removed by washing sections with wash buffer three times for 5 min each. Then each section was covered with 1-3 drops Signal Stains Boost Detection Reagent (HRP) and incubated in a humidified chamber for 30 min at room temperature. The Signal Stain Boost Detection Reagent (HRP, Rabbit) was equilibrated to room temperature before use. Next, section was washed three times with wash buffer for 5 min each. One drop (30µl) of SignalStain DAB Chromogen Concentrate was added to 1 ml SignalStain DAB Diluent and mix well before use. 100-400µl Signal Stain DAB was applied to each section 5-10 min followed by immersion of sections in dH2O and subsequent sections counterstaining with hematoxylin. The sections were washed in dH2O two times for 5 min each, followed by dehydration of section. **Dehydrate sections:** Sections were incubated in 95% ethanol two times for 10 seconds each, repeated in 100% ethanol, followed by the incubation of sections two times for 10 secs. This was then repeated in xylene and incubated two times for 10 each section. Finally, sections were mounted with coverslips and mounting medium. Histoscore for positive stains was evaluated as following: (1 × % weakly stained cells) + (2 × % moderately stained cells) + (3 × % strongly stained cells) [22].

**Statistical analysis:** Data were presented as

Mean ± SEM. Differences in experimental rat groups were compared with One-way ANOVA followed by Tukey post-hoc test, Pre and post-rectal temperatures were compared with Paired t-test.  $P < 0.05$  was regarded as statistically significant. Graph pad 5 software package (USA) was used for the analysis.

**Results**

**Oxidative stress parameters (SOD, CAT, GSH and MDA) of salt-loaded rats exposed to high environmental temperature**

Plasma CAT activity and GSH concentration were lower in rats fed a high salt diet alone ( $p < 0.05$ ,  $p < 0.05$ ) and in rats exposed to high environmental temperature alone ( $p < 0.05$ ,  $p < 0.05$ ) compared to control rats. In addition, plasma MDA concentration was higher in both experimental rats compared to control ( $p < 0.01$ ,  $p < 0.001$ ). Similarly, plasma CAT activity and GSH concentration were lower in rats exposed to the combined environmental factors ( $p < 0.05$ ,  $p < 0.01$ ), with elevated plasma MDA concentration ( $p < 0.001$ ) compared to control rats. However, plasma MDA concentration was higher in rats exposed to the combined environmental factors ( $p < 0.01$ ) than in rats exposed to high either environmental temperature alone or in rats fed a high salt diet alone. This indicates a synergistic effect of the two environmental factors on circulatory MDA.

**Table 1.** Effect of HET on oxidative stress parameters in salt-loaded rats.

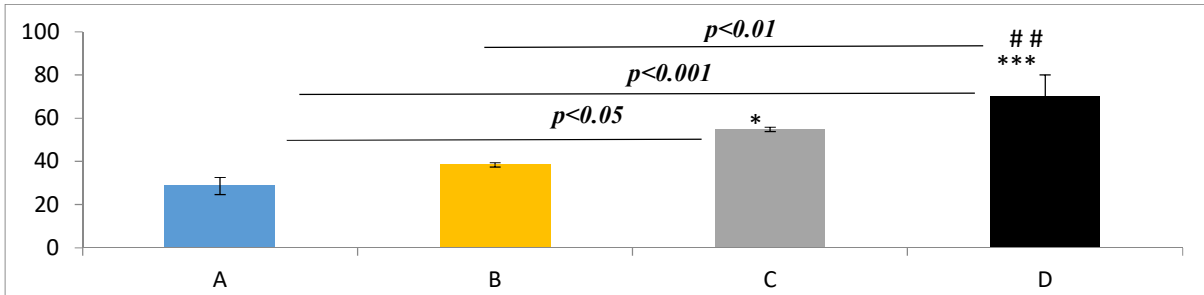
Groups	SOD (U/mg of protein)	CAT (U/mg of protein)	GSH (µmol/ml)	MDA (∞mol/ml)
Control	945.4 ± 106	467.7 ± 15.9	17.0 ± 1.1	3.0±0.5
Salt	526.5 ± 54.3	239.1 ± 40.6*	10.6 ± 0.3*	7.3±0.5**
Heat	502.7 ± 97.6	260.3 ± 55.0*	10.7 ± 0.9*	13.5±0.5***
Salt+heat	553.7 ± 61.7	253.5 ± 61.4*	9.2 ± 1.4**	18.9±1.3***###

SOD: super oxide dismutase; CAT: catalase; GSH: reduce glutathione; MDA: Malondialdehyde; \* $p < 0.05$ , \*\* $p < 0.01$  \*\*\* $p < 0.001$  vs Control, ### $p < 0.001$  vs Salt, #### $p < 0.001$  vs Heat presented as Mean ± SEM (n=8). HET: high environmental temperature.

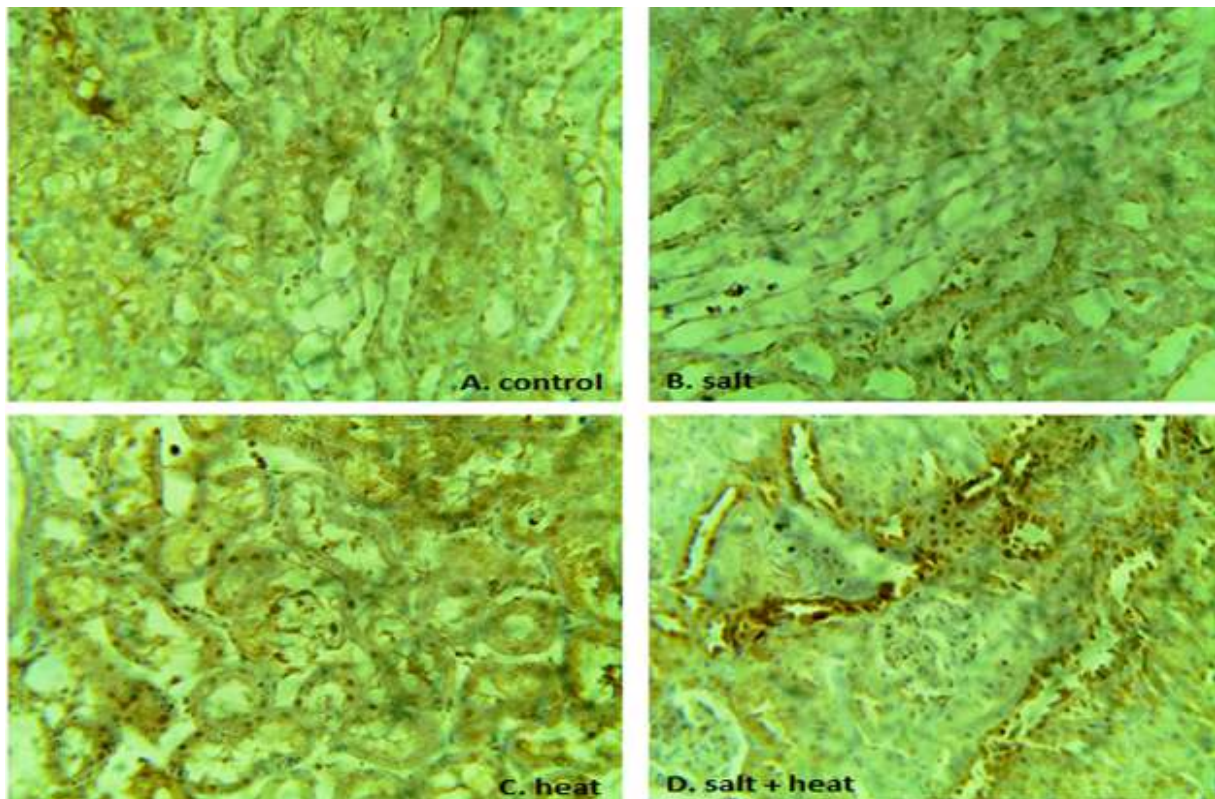
Meanwhile, plasma SOD activity although tended towards a decline in all the experimental rats was not significantly different compared to control rats (Table 1). The decreased plasma CAT activity, GSH concentration and increased MDA above are indicative of increased oxidative stress in all the experimental rats.

***p38 MAPK expression in the kidney of salt-loaded rats exposed to high environmental temperature***

p38MAPK was weakly expressed in the kidneys of control rats (A) and in rats fed on high salt diet alone (B), but was moderately expressed in the kidneys of rats exposed to HET



**Figure 1a. Percentages p38 MAPK expression in renal tubules.**



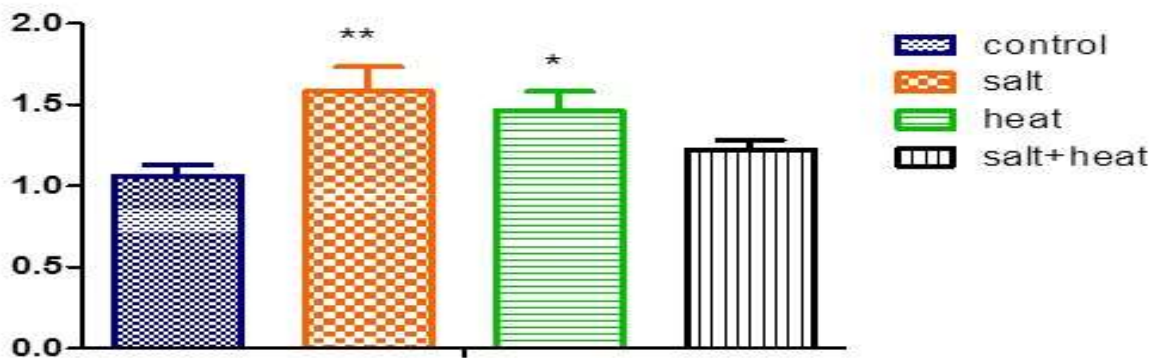
**Figure 1b. Immunohistochemistry of renal tubules showing the expression of p38 MAPK in the kidneys of experimental rats:** control rats (A) were fed with normal diet (0.3% NaCl), salt-loaded rat (B) were fed with high salt diet (8% NaCl), heat-exposed rats were fed with normal diet but exposed to HET (C), salt-loaded + heat-exposed rats (D) were fed with high salt diet (8%NaCl & exposed to HET). The expression of p38 higher in salt + heat rats was higher than the expression in salt-loaded rats (B) as well as in the heat-exposed rats (C). (Magnification, X 400). Formalin-fixed and paraffin embedded rat kidneys labeled with Anti-p38 MAPK Polyclonal Antibody, Unconjugated (Cell Signaling Technology, USA) at 1:200 followed by conjugation to the secondary antibody and DAB staining. Data presented as mean ± SEM (n=5).

alone (C) ( $p < 0.05$ ) and strongly expressed in the kidneys of rats exposed to combined environmental factors (D) compared to control ( $p < 0.001$ ). Furthermore, the expression of p38 MAPK in the kidneys of rats exposed to the combined environmental factor was stronger ( $p < 0.01$ ) than those of the salt group, indicating a possible synergistic interaction between HSD and HET on p38 MAPK expression (Figure 1a & b).

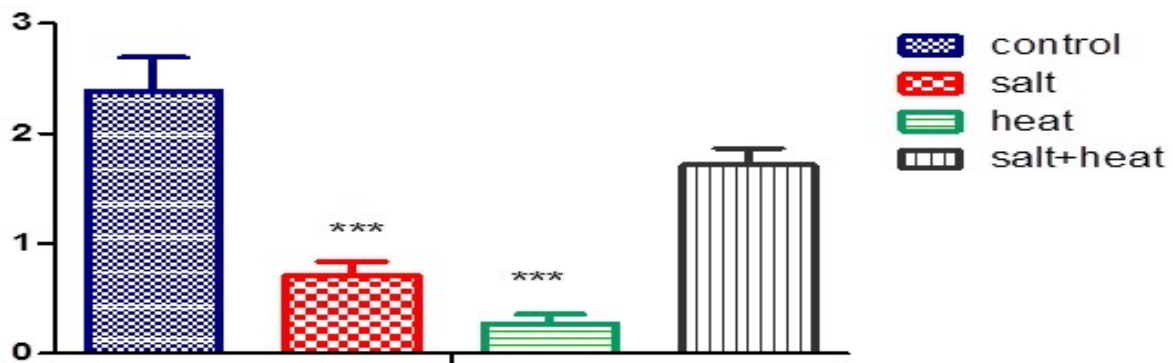
**Circulatory HSP70 and nitric oxide in salt-loaded rats exposed to high environmental temperature**

In comparison with control, plasma HSP70

(ng/ml) was significantly higher in rats fed a high salt diet alone ( $1.1 \pm 0.1$  vs  $1.6 \pm 0.2$ ,  $P < 0.01$ ) and in rats exposed to high environmental temperature alone ( $1.1 \pm 0.1$  vs  $1.5 \pm 0.1$ ,  $P < 0.05$ ), but unchanged in rats exposed to both factors ( $1.1 \pm 0.1$  vs  $1.2 \pm 0.1$ ,  $P > 0.05$ ). However, plasma NO ( $\mu\text{M}$  unit) was significantly lower in rats in rats fed a high salt diet alone ( $2.4 \pm 0.3$  vs  $0.7 \pm 0.1$ ,  $P < 0.001$ ) and in rats exposed to high environmental temperature ( $2.4 \pm 0.3$  vs  $0.3 \pm 0.1$ ,  $P < 0.001$ ) compared to control, but unchanged in rats exposed to a combination of high environmental temperature and high salt diet ( $2.4 \pm 0.3$  vs  $1.7 \pm 0.2$ ,  $P > 0.05$ ).



**Figure 2.** Heat shock protein 70 (ng/ml); \* $P < 0.05$ , \*\* $P < 0.01$  vs control; Data presented as Mean  $\pm$  SEM (n=8).

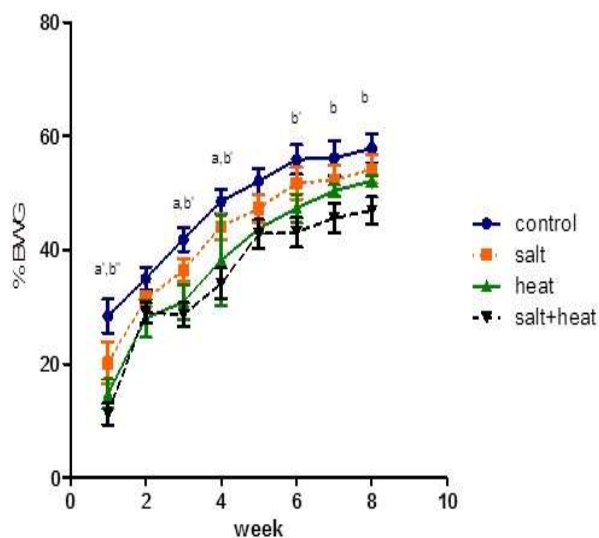


**Figure 3.** Nitric Oxide ( $\mu\text{M}$  unit); \*\*\* $P < 0.001$  vs control. Data presented as mean  $\pm$  SEM (n=7).



### Body weight gain in experimental rats of salt loaded rats exposed to high environmental temperature

High environmental temperature alone and combined with high salt diet significantly ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ) retarded weekly body weight gain in the experimental rats (Figure 4).



**Figure 4.** BWG: Body Weight Gain; <sup>a</sup> $p < 0.05$ , <sup>a'</sup> $p < 0.01$  vs heat; <sup>b</sup> $P < 0.05$ , <sup>b'</sup> $p < 0.01$ , <sup>b''</sup> $p < 0.001$ ; Data presented as Mean  $\pm$  SEM (n=8).

### Discussion

High environmental temperature has been described as a silent killer [23], while high salt diet consumption was reported to be responsible for 1.65 million cardiovascular-related global deaths in 2010 [24]. Our previous study showed that high environmental temperature increased the severity of hypertension in male Sprague-Dawley rats fed a high salt diet by increasing salt retention [5]. This current study attempted to elucidate the plausible role played by oxidative stress mechanism and apoptotic markers in driving this outcome. First, we observed that high

environmental temperature combined with high salt diet over a long-term increased the product of oxidative cellular damage, MDA, in blood circulation, with associated increase in p38 MAPK expression in the kidneys of our experimental rats. Next, circulatory NO and HSP70 were unaltered by high environmental temperature in rats fed with high salt diet. Lastly, our results demonstrated that high environmental temperature significantly slowed down weekly body weight gain in rats fed with high salt diet.

Oxidative stress is an important mechanism underlying the pathophysiology of several disease conditions, including cardiovascular disorder [25] and kidney injury [26]. It occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidant defense mechanisms production. The antioxidant defense system, including SOD, CAT, glutathione peroxidase (GPx) and GSH, protect cells against the deleterious effects of ROS namely Superoxide radicals ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $\bullet OH$ ). The depletion of these antioxidants results in progressive oxidative damage to cellular macromolecules, including lipid, proteins and DNA in biological organs. Oxidative damage to membrane lipid in turn increases circulatory MDA which is considered as a marker of lipid peroxidation and cellular damage [27]. In addition, MDA is a highly reactive agent with the ability to attack different cellular macromolecules, including amino acid and sulfhydryl moiety of proteins. This could promote the alterations of biochemical properties of these biomolecules, and subsequently results in the loss of functional protein, modifications of enzymes and carriers proteins, distortion of cytoskeletal units and DNA bases, consequently leading to cell death [28].

In this current study, rats chronically fed with HSD alone or exposed to prolonged HET alone had significantly depleted plasma CAT activity and GSH concentration with raised MDA concentration compared to control rats. Similarly, plasma CAT activity and GSH concentration were depleted in rats fed a high salt diet in combination with exposure to high environmental temperature. However, plasma MDA concentration in rats exposed to the combined environmental factors was significantly higher than the level seen in rats exposed to either of the factors. First, these findings indicate that oxidative stress resulting from prolonged high salt diet consumption alone was comparable to that caused by prolonged exposure to high environmental temperature alone. But most importantly, this present result suggests that interaction between high salt diet and high environmental temperature exacerbates lipid peroxidation and cellular damage as evident by the synergistic rise in circulatory MDA concentration in our experimental rats. In agreement with our findings, earlier studies also demonstrated the contribution of high salt diet [29] and environmental heat [30] to oxidative stress in health and diseases. Meanwhile, previous studies have shown that oxidative stress caused by environmental stressor activates p38 MAPK signaling pathway to mediate DNA damage and cell death [11], while HSP70 is induced to offer protection against apoptotic cell death [8]. Again, p38 MAPK has been reported as a key driver of HSP70 induction [31]. Most importantly, a protective relationship is reported to exist between the activation of p38 MAPK and the induction of HSP70 [32]. Interestingly, the interaction between HSD and HET in our present study significantly increased the expression of p38MAPK in the renal tubules of our experimental rats, with

some observed synergistic effect as seen in the circulatory MDA. The renal expression of p38 MAPK was also increased moderately by exposure to HET but not HSD alone. This is partly in agreement with a study by Hao et al. [33] which showed that thermal stress increases the phosphorylation and the expression of p38 MAPK similar to the action of high-salt diet [34]. This is indeed germane, since p38 MAPK pathway plays key role in the pathophysiology of hypertension [35], renal failure [36] and DNA damage [11]. The molecular actions of MAPK in the kidney [35] and other organs results from its ability to upregulate specific inflammatory cytokines such as IL-6, IL-8, and TNF $\alpha$  in several biological contexts, including kidney damage, myocardial injury [37], cardiomyocytes apoptosis and spontaneous hypertension in stroke prone rats [13]. These actions, in addition with the potential of p38 MAPK to activate SGK1 diet [34], possibly contributed to the increased salt retention caused by the interaction between HSD and HET in our previous study [5]. Specifically, SGK1 increases epithelial sodium channel (ENaC)-mediated Na<sup>+</sup> transport by a number of mechanisms in the kidney, including increased apical membrane localization of ENaC, inhibition of ENaC degradation and stimulation of ENaC transcription [38]. This is indeed critical given the role of ENaC in salt-sensitive hypertension. Meanwhile, circulatory HSP70 is a reliable indicator of presence of chronic stress, released intracellularly and extracellularly by several environmental stress factors, including thermal and salt-induced stress [7]. Generally, the presence of HSP offers protection against programmed cell death via increase correction of folding of regulatory proteins, reduced activation of p38 kinase [39], inhibition of apoptotic signaling pathways in chronic

diseases [31] and the suppression of caspase-3 activation critical to cell death [40]. But in addition, HSP70 confers thermotolerance on cells previously exposed to hot environment [42], protect the liver against oxidative stress [43], enhances nitric oxide production [9] and increases the chances of animal survival.

In this current study, HSP70 induction was higher in rats exposed to high environmental temperature alone and in rats fed with high salt diet alone compared to control rats. This result is in tandem with earlier investigations which reported similar increase in HSP70 induction by heat stress [44] and high dietary salt [7]. But combined together, HSD and HET did not increase the induction of HSP70 in this present study. This implies a possible antagonistic effect of interaction between HSD and HET on HSP70 induction and the consequent abrogation of the potential protective activity of HSP70 on vital organs. This is supported by studies which showed that the inhibition of HSP70 resulted in the withdrawal of its cytoprotective influence [33], whereas the inhibition of p38 MAPK mitigated the physiological impact of stress [40]. Therefore, the synergistic increase of renal p38 MAPK expression with associated unaltered HSP70 portends double risks in rats exposed to HSD combined with exposure to HET. This thought is supported by the observed retarded body weight gain in this group of rats. Furthermore, in comparison to control rats, circulatory NO was lower in rats fed a HSD alone and in rats exposed to HET alone, with associated rise in HSP70, but was unchanged in rats exposed to both factors with associated unchanged HSP70. The depleted circulatory NO with the converse rise in HSP70 supports a study which suggest that NO inhibition resulted in HSP70 induction [45]. Invariably, the absence of NO inhibition in rats exposed to the combined environmental

factors possibly contributed to the unchanged HSP70 albeit the presence of the dual environmental stressors.

The main limitation of our study includes inadequate resources to evaluate the level of phosphorylation of p38 MAPK expressed in kidneys of the experimental rats. This knowledge could further provide information on the activation of this important cellular marker. In addition, Lack of investigation of inflammatory cytokines upregulated by p38 MAPK was also another limitation in this study.

### **Conclusion**

Overall, our study suggests that prolonged exposure to high environmental temperature may worsen the cardio-renal outcome of long-term consumption of high dietary salt by way of escalating renal expression of p38 MAPK and promoting oxidative cellular damage, with conversely unchanged circulatory HSP70 and NO. These change possibly contributed to the severity of hypertension caused by the two environmental factors as presented in our earlier work. In addition, interaction between high salt diet and high environmental temperature may also exert negative impact on growth. It is not however clear whether or not the increased expression of p38 MAPK was a compensatory response to the unaltered HSP70 induction in rats exposed to the combined environmental stress.

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**Ethical statement:** *The study was conducted in accordance with the ethical approval of the University Ethics Committee. (Date: 24/10/2019; Decision number: 2019/217).*

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## Comparison of fully automatic analyzer and manual measurement methods in sperm analysis and clinical affect

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### ABSTRACT

**Aim:** To investigate the clinical effect of the computer-aided sperm analyzers (CASA) by comparing the low sperm concentration semen samples evaluated by CASA with the sperm count performed on Makler Counting Chamber (MC) as a manual method.

**Methods:** Semen samples were taken from 184 patients coming to our clinic were evaluated with CASA (SQA-V Gold sperm analyzer, MES Medical Electronic Systems Ltd. Caesarea Industrial Park, IL 3088900, UK) and MC (Makler Counting Chamber, Sefi-Medical Instruments Ltd., Haifa, Israel). Samples were divided into two groups as samples containing sperms and samples without sperms, according to the CASA results.

**Results:** There was a very high correlation between the two measurement methods ( $\rho = 0.982$ ) and regression analysis formula was  $y = 1.042x - 0.104$ . No sperm was detected in CASA in any of the samples identified to have no sperm in MC. However, when patients who were identified with no sperm in their CASA measurements ( $n = 51$ ) were analyzed with MC, 29 patient samples (56.9%) had an average of  $0.23 \pm 0.35 \times 10^6$  /mL sperm.

**Conclusion:** CASA's used in routine semen analysis provide a great convenience in measuring sperm count, compared to manual methods and provide highly correlated results. Manual verification of samples can be recommended since the samples diagnosed with azoospermia provided different results with a manual method in our study.

**Keywords:** Infertility, azoospermia, computer-aided sperm analyzers, method comparison.

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### Introduction

Infertility is a potentially life-changing diagnosis for couples who are trying to

conceive. It can be defined as the condition of not being able to conceive despite regular unprotected intercourse for at least 12 consecutive months [1,2]. Male factor is suspected in approximately half of the cases [3]. The most common and precise diagnostic step in male infertility is semen analysis. Semen analysis helps to investigate male infertility and provides basic data on the contribution of the male factor for an infertile couple [4]. It also

helps to identify reversible medical conditions that can affect fertility [5]. The subjectivity of the evaluation and interpersonal variation of sperm concentration and motility are the most significant limitations of this technique [6]. The diagnostic and prognostic effectiveness of semen analysis is correlated with strict compliance to the guidelines recommended by the World Health Organization. Neubauer slide (NS), Makler counting chamber (MC), spectrophotometric methods and fully automated (or computer-aided) sperm analyzers (CASA) can be used for sperm counting [3,6,7]. Spermatozoa motility, morphology and concentration can be analyzed simultaneously on modern CASA systems but such assessments are not as reliable as traditional methods (such as NS or MC) [8,9]. Computerized systems such as CASA, are more convenient for analyzing complex parameters such as sperm motility and offer an objective and fast method for semen analysis. CASA uses a microscope, camera and computer software for sperm motility analysis [7,8]. In traditional semen analysis methods, sperm cells in the semen placed on a slide such as NS or MC are counted on the microscope [10]. The complete absence of sperm in semen analysis is defined as azoospermia [6,11]. Azoospermia is the definition of the semen rather than the basis of diagnosis and treatment or the cause of sperm absence [10]. However, azoospermia is not the case even if there is a single sperm in the semen. Even the case of a single sperm can affect the treatment and this becomes a condition of subfertility rather than infertility [12]. Although seemingly simple, the diagnosis of azoospermia is complicated by many factors, such as significant errors associated with counting a small number of spermatozoa, a large number of microscopic fields to be examined, and the difficulty of examining debris-loaded sperm

pellets. It is recommended to examine fixed but non-centrifuged samples to overcome these situations [10].

In the widely used CASA, patients with a very low amount of sperm in their semen who are diagnosed with azoospermia is a frequent situation. This study investigates the clinical effect of CASA by comparing the low sperm concentration semen samples evaluated by CASA with the sperm count performed on MC.

### **Materials and Methods**

The study was approved by Bolu Abant Izzet Baysal University, Clinical Research Ethics Committee, decision number 2020/60, dated 07/04/2020. Semen samples were taken from 184 patients, who applied and gave written consent to the male infertility laboratory. The samples were macroscopically confirmed to be semen samples. Semen samples were obtained through masturbation by dry method in sterile containers. Samples were analyzed after liquefaction in the incubator (Heraeus, Thermo Electron Corporation, Langensfeld, Germany) for about 30 minutes at 37°C and thorough mixing. Samples which were less than 1 ml and more than 4 ml, samples that were not treated with liquefaction within 30 minutes and samples showing hyperviscosity were excluded from the study [10,13-15]. Fresh semen samples were evaluated without dilution and processing on the samples included in the study. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Semen samples were analyzed with SQA-V Gold sperm analyzer (SQA-V Gold sperm analyzer, MES Medical Electronic Systems Ltd. Caesarea Industrial Park, IL 3088900, UK)



for CASA method, and MC (Makler Counting Chamber, Sefi-Medical Instruments Ltd., Haifa, Israel) for manual method. Samples were taken blindly from the same pool to be evaluated blindly at the same time and transferred to the device for CASA and to the microscope with MC for manual method. In sperm analysis, the sperm concentration was determined as  $10^6/\text{mL}$ . Motility in sperm analysis was evaluated as; progressively motile, non-progressively motile and immotile. Motility rates were given as a percentage of total sperm count. Samples were divided into two groups as samples containing sperms and samples without sperms, according to the CASA results. The manual microscopic method was performed in compliance with the standard protocol in the WHO 2010 guideline [6,10]. Automated analysis was performed by using the laboratory-based CASA system, SQA-V Gold sperm analyzer. Automatic semen analysis was performed in accordance with the protocol of the manufacturing company. In summary, samples were mixed thoroughly and inserted in the device's electro-optic chamber with a capillary for CASA counting. Sperm counts and movements are reported automatically after the data are analyzed

through special algorithms in the computer system by translating the light-beams into electrical signals. The measurement range for the sperm concentration of the SQA-V Gold sperm analyzer was specified as 0-700  $10^6/\text{mL}$  by the manufacturer.

**Statistical analysis**

Statistical analysis of the data was performed through the SPSS program (version 17.0, SPSS Inc., Chicago, IL, USA). The conformity of the numerical values to normal distribution was evaluated through the Kolmogorov-Smirnov test. Descriptive data were presented as mean  $\pm$  standard deviation and median (1<sup>st</sup> - 3<sup>rd</sup> quarter). Wilcoxon-rank test was used in the comparison of dependent variables after it was determined that the data did not conform to normal distribution. McNemar test was used in the comparison of paired nominal data. Passing-Bablok regression analysis and Spearman correlation analysis were used to evaluate the compatibility between the two methods.  $p < 0.05$  was considered to be statistically significant.

**Results**

CASA and MC semen analysis results are shown in Table 1. The median sperm count values according to CASA and MC were 16.4

**Table 1.** Comparison of CASA and MC semen analysis results.

Parameters	CASA		MC		p value*
	$\bar{x} \pm \text{SD}$	Md (Q1-Q3)	$\bar{x} \pm \text{SD}$	Md (Q1-Q3)	
<b>Sperm number (<math>\times 10^6/\text{mL}</math>)</b>	29 $\pm$ 33.9	16.4 (0-46.8)	28 $\pm$ 31.8	16 (0.2-40)	0.066
<b>Immotile (%)</b>	37.5 $\pm$ 33.3	35 (0-68.8)	51.3 $\pm$ 31.7	50 (25.3-79)	<0.001
<b>Non-progressive motile (%)</b>	10.7 $\pm$ 10.9	10 (0-17)	19.2 $\pm$ 17.1	20 (0.8-30)	<0.001
<b>Progressively motile (%)</b>	24.4 $\pm$ 26.6	15 (0-44.8)	16.1 $\pm$ 20.2	10 (0-30)	<0.001

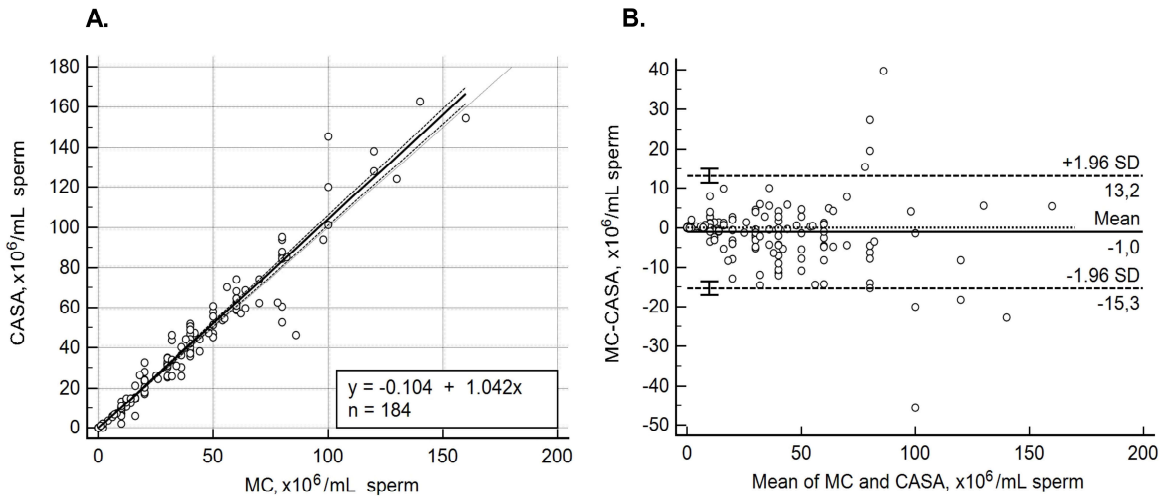
$\bar{x} \pm \text{SD}$ : mean  $\pm$  Standart deviation, Md (Q1-Q3): Median (1<sup>st</sup>-3<sup>rd</sup> quartile), \*: Wilcoxon signed-rank test. CASA: computer-aided sperm analyzers. MC: Makler Counting Chamber.

(0.0 - 46.8) and 16.0 (0.2 - 40.0), respectively, and there was no statistical difference between the two values ( $p = 0.066$ ). There was a very high correlation between the two measurement methods ( $\rho = 0.982$ ) and the Passing-Bablok regression analysis formula was  $y = 1.042x - 0.104$  (Figure 1). Comparison of groups with and without sperm according to CASA and MC is shown in Table 2. No sperm was detected in CASA in any of the samples identified to have no sperm in MC. However, when patients who were identified with no sperm in their CASA measurements ( $n = 51$ ) were analyzed with MC, 29 patient samples (56.9%) had an average (min-max) of  $0.23 \pm 0.35$  (0.1-2.0) $\times 10^6$  /mL sperm.

**Discussion**

In our study where sperm concentrations in semen analysis were evaluated, the semen samples that arrived at our laboratory were examined with CASA and MC and being the diagnostic criteria in the diagnosis of azoospermia, only sperm concentrations were compared. In the measurements performed by CASA and MC, there was a high correlation with regards to sperm concentration. In 57% of the samples that would be diagnosed as azoospermia through CASA, the presence of sperm was detected through MC.

In studies have shown high correlations between CASA and manual methods with regards to sperm parameters [16,17]. Kose et al.



**Figure 1.** Passing-Bablok regression (A) and Blant-Altman (B) plots of CASA and MC sperm numbers.

**Table 2.** Comparison of the groups with and without sperm according to CASA and MC.

Parameters	No-sperm in MC	Sperm in MC	P value*
No-sperm in CASA	22 (%43.1)	29 (%56.9)	<0,001
Sperm in CASA	0 (%0)	133 (%100)	

\*McNemar's test. CASA: computer-aided sperm analyzers. MC: Makler Counting Chamber.

found a correlation of 0.84 between methods in terms of sperm concentration [16]. Similarly, Lammers et al. showed that there was a 0.95 correlation between various CASA methods and manual method in terms of sperm count [17]. In parallel to the literature, a correlation of 0.98 was found between CASA and the manual method in our study.

Wang et al. [7] stated that sperm motility and morphology were associated with the time until

natural pregnancy, while sperm motility might be less predictive. Gnoth et al. [12] named the absence of sperm as azoospermia and classified the prolonged time to conceive as subfertility. In our study, sperm count was evaluated and it was observed that when the same samples were examined with two different methods with regards to azoospermia, sperms could be found in the samples that were reported as azoospermia with CASA when analyzed in detail with the manual method.

Bjorndahl et al. [18] prepared a guideline to journals for better sperm analysis evaluations. They developed criteria for evaluation of the general analysis, concentration, motility, morphology, sperm viability, other findings and analysis data in the evaluation of semen analysis. Our study fulfilled all seven criteria of sperm concentration evaluation in this guide.

As a result of the improvements in CASA systems in parallel with the development of hardware, the capability to gradually analyze the concentration of moving spermatozoa by using fluorescent DNA stain and a tail detection algorithm in addition to sperm concentration provided a superiority over manual methods in motility measurement [9,19]. However, in our study, different clinical findings were shown in semen analysis in very low concentrations which could not be measured by CASA. Detecting sperms in 57% of the samples that cannot be measured by CASA through manual evaluation demonstrates the importance of verification of sperm analysis in very low concentrations with a manual method, despite the current improvements and superiority of CASA over manual methods in certain parameters.

Although it is known that sperm parameters can be extremely variable even if the sperm analysis results of the same individuals do not differ significantly at various times, it is stated that

sperm concentration analysis is one of the most reliable methods [20]. Variability is even more important at low sperm concentrations, and our study recommends the analysis of these low-concentration samples with more than one method.

Currently, in clinical laboratories worldwide, a semen analysis is still based on a manual microscopy method. However, some of the major disadvantages of this technique are that it is labour-intensive, subjective, laboratory-based, and time-consuming. Although partial automation of routine semen analysis with CASA is adopted in clinical use, it is reported in studies that it is still in the development phase to receive wider acceptance [15,19,21,22]. Our study on the other hand, emphasizes that a manual microscopy method is required clinically, specifically in approaching the azoospermia cases.

### **Conclusions**

CASA's used in routine semen analysis provide a great convenience in measuring sperm count, compared to manual methods and provide highly correlated results. However, in the evaluation of azoospermia, it is known that the presence of even a single sperm in the sample may change the clinic and treatment. Manual verification of samples can be recommended since the samples diagnosed with azoospermia provided different results with a manual method in our study.

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**Conflict of Interest:** *The authors declare that they have no conflict of interest.*

**Ethical statement:** *The study was conducted in accordance with the ethical approval of the Bolu Abant Izzet Baysal University Ethics Committee. (Date: 07/04/2020; Decision number: 2020/60).*

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## Utility investigation of automated techniques in hematopoietic progenitor cell count and viability assessment in the Good Manufacturing Practice (GMP) setting

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### ABSTRACT

**Aim:** To compare our parameters as regards: i) cell count via two different automated cell count techniques, and ii) viability via automated trypan blue exclusion and 7-aminoactinomycin D (7-AAD) staining.

**Method:** We used the trypan blue exclusion technique and an automated cell counter and for viability testing, and the trypan blue exclusion technique and the 7-AAD evaluation by flow cytometry. The trypan blue exclusion and the radio frequency techniques were used for automated cell counting. Flow cytometric analysis was performed by evaluating the yielded cellular products for 7-AAD uptake during the cell count of CD34+ cells.

**Results:** The mean values for cell count were estimated as  $3.44 \pm 1.22 \times 10^6/\text{ml}$  (range,  $2.48-5.71 \times 10^6/\text{ml}$ ) and  $4.14 \pm 1.94 \times 10^6/\text{ml}$  (range,  $1.77-7.43 \times 10^6/\text{ml}$ ) for the trypan blue exclusion and radio frequency techniques, respectively. Additionally, the mean values for viability analyses via the automated trypan blue exclusion and 7-AAD were  $93.38 \pm 6.09\%$  (range,  $79.00-98.00\%$ ) and  $99.49 \pm 0.60\%$  (range,  $98.40-100.00\%$ ), respectively.

**Conclusions:** Our study has responded to two fundamental questions: whether the results of both of the automated techniques for cell count correspond with each other, and whether the results of the automated viability assessment conform those of the 7-AAD technique during the manufacturing processes of cellular therapy products intended for clinical use. Even though we have the opportunity to use the hemocytometer in our laboratory setting, the automated trypan blue exclusion technique gives cell count results in concordance within the range of the expectations of our Quality Management System (QMS).

**Keywords:** 7-AAD, automated cell count, cellular therapy, quality, safety, viability.

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## Introduction

Promising to be first-line therapy for many kinds of different diseases in the future, cellular therapies are gradually expanding as a treatment option in many clinics. Cellular therapies can be bluntly defined as *in vitro*-manipulated human cells which require certain safety and quality parameters as prerequisites – two of which are cell count and viability– at the time of release from the laboratory to the clinic. Cell counting techniques can either be performed manually (e.g. hemocytometer) or by the use of automatic devices which are operated by certain principles such as the automated trypan blue exclusion [1,2] and radio frequency [3,4]. Viability assessment is also possible via the trypan blue exclusion technique [5]. Also some manual methods– i.e. acridine orange, eosin staining can be used for viability detection [6]. Alternatively, the viability dye 7-aminoactinomycin D (7-AAD) can also be used to determine the number of viable CD34+ cells [7,8]. In this manner, automated techniques present themselves to be utile in performing both cell count and viability estimation, and when necessary being able to give both results realtime.

Previously, researchers made various comparisons of manual and automated cell count and viability techniques in different cell types [2,5,6,9-11]. However, none of these studies show comparison between automatic and/or manual cell count and viability techniques by means of hematopoietic progenitor cells. In this study, we aimed to compare our parameters as regards: cell count via two different automated cell count techniques, and ii) viability via automated trypan blue exclusion and 7-AAD staining. As noticed, studies practicing the comparison of different automated cell counting methods on human stem cells have not yet been performed.

Under the scope of the current Good Manufacturing Practices (cGMP) activities at Ankara University Stem Cell Institute Tissue and Cell Manufacturing Center, we obtained purified CD34+ hematopoietic stem cell products intended for use in patients mainly suffering from severe combined immune deficiency (SCID). We had previously reported our local experience with the CliniMACS (magnetic-activated cell separation system) in hematologic malignancies and immune failure disease. There, we evaluated our CliniMACS CD34+ cell enrichment process by revealing absolute cell count and viability besides other parameters for the end products [12]. This study has two objectives: one is to determine whether the two automated techniques' results for cell count match each other, and whether the automated cell counter results for viability match those of the 7-AAD technique during the manufacturing processes of cellular therapy products intended for clinical use. This is the first time that hematopoietic progenitor cell count and viability testing are compared between different automated techniques in order to suggest automated cell counters as simple-use devices with the ability to produce reliable and timely results.

## Materials and Methods

As defined in the study of Kilic et al. [12], the apheresis products were transferred from Ankara University School of Medicine İbn-i Sina Hospital Therapeutic Apheresis Unit Center to our Tissue and Cell Manufacturing Center, within a sterile container, with the facility to transport at a stable temperature recorded by a data logger [12]. The records containing the results of complete blood count (CBC), CD34+ cell enumeration and viability obtained by flow cytometric evaluation were accompanied with each sample. The study was

approved by the Turkish Ministry of Health Turkish Medicines and Medical Devices Agency, with the approval number 2014/2 for manufacturing of human medical products. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was collected from each subject.

Samples were used to perform 8 separate CliniMACS CD34+ enrichment process cycles. The enrichment process was carried out as described in the CliniMACS® User Manual (CliniMACS User Manual), and was followed in accordance with the study of Leong et al. [9]. The CD34+ cell selection technique is used to deplete T cells from collected human-based cell products before allogeneic HSC transplantation. The CD34+ cells can be separated by various devices, one of which is the CliniMACS (Miltenyi, Biotec, GmbH, Bergish, Gladbach, Germany) [9,12-14]. The CliniMACS CD34+ cell enrichment process is performed within the Quality Management System of our Tissue and Cell Manufacturing Center, as described by Kilic et al. [12]. Cell count and viability are the most critical release criteria besides other tests, such as sterility and endotoxin test, for each CD34+-enriched end product. In the 8 cell count processes included in this study, we used the trypan blue exclusion technique and an automated cell counter for cell count, and for viability testing, the trypan blue exclusion technique and the 7-AAD evaluation by flow cytometry [12].

### **Cell count**

#### ***Automated cell count via trypan blue exclusion***

In the beginning, the trypan blue exclusion test was performed by the use of the TC20™ Automated Cell Counter [1]. This device provides cell counts within the range of  $5 \times 10^4$

to  $1 \times 10^7$  cells/ml [2]. Twenty microliters of the 0.04% trypan blue staining solution and 20  $\mu$ L of each sample were mixed within the test tube. Ten microliters taken from this mixture was pipetted and placed on the counting chamber. If the cell number exceeded  $1 \times 10^7$  cells/ml, the samples were diluted with saline solution at the ratio of 1:9, and the count was repeated thereafter [6].

#### ***Automated cell count via radio frequency***

The radio frequency principle was used as the second automated cell count technique (Sysmex Europe GmbH. Sysmex XN-3000). Devices operating under such principle are only certified for testing blood samples, hence do not guarantee use of other bodily fluids. The Sysmex XN-3000 is a fully automated complete blood count (CBC) hematology analyzer including 6-part differential count. This analyzer differentiates white blood cells (WBC) and tests 28 standard diagnostic CBC parameters. The XN-3000 processes 200 samples/hour and includes the SP-10 slidemaker/stainer for reflexive slide preparation [15]. Cells in an aliquot (1 cc) of each of the end products were automatically counted with the Sysmex XN-3000 Automated Cell Counter.

### **Viability**

#### ***Automated cell counter via trypan blue exclusion***

Viability was assessed via an automated cell counter, using the trypan blue exclusion technique [1]. The automated device was used after preparation of samples as explained in section “2.1.1 Automated Cell Count via Trypan Blue Exclusion”. In this technique, the automated cell counter detects the dead cells, which are instantly stained with the trypan blue, within the total cell population. The viability of



the cells is displayed on the screen in terms of percentage of viability.

#### **The 7-aminoactinomycin dye (7-AAD) method**

Flow cytometric analysis was performed at Ankara University Hematology Laboratory as explained in the studies of Varan et al. [6] and Kilic et al. [12]. Briefly, the yielded cellular products were evaluated for 7-AAD uptake during the flow cytometric count of CD34+ cells. At the end of the CD34+ enrichment process, the end products were evaluated for cells expressing CD34 and also for CD45, CD3 CD56, CD19, and CD14 to further characterize the cell content of the product. Cell viability was checked for each sample using the viability dye 7-AAD, and all counts were reported in terms of viable cells. The Kaluza software ver2.1 (Beckman Coulter Miami, USA) was used to analyze the collected data using the Navios 3L10C device (Beckman Coulter Miami, USA). CD34+ cell counts were calculated according to the single platform ISHAGE protocol [16]. The statistical data of the charts were retrieved from the statistical results of the report and the ratio of dead cells (cells stained with 7-AAD) was determined. The percentage of living cells was determined by subtracting the percentage of dead cells from 100.

#### **Statistics analysis**

Statistical analysis was performed by using the SPSS 22 version package program. Correlation between cell count and viability results,

obtained from different methods, were tested using the 2-tailed Pearson correlation analysis ( $p=0.05$ ).

#### **Results**

An example of three consecutive cell count and viability results obtained by our automated cell counter is presented in Figure 1.

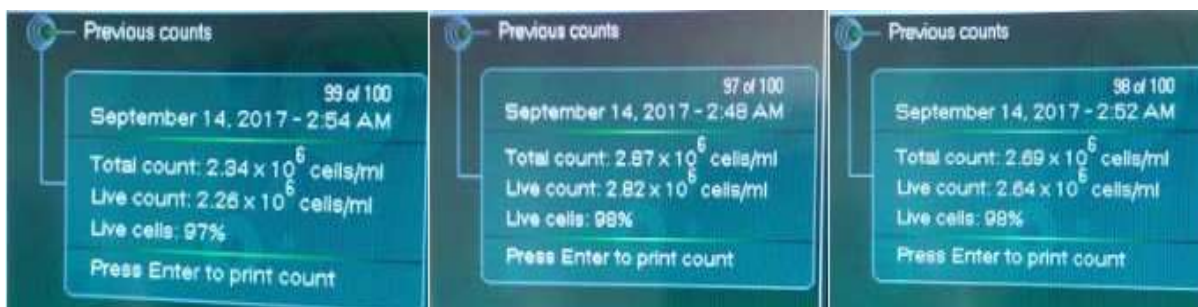
The distribution of the cells was checked in two-dimensional dot plot graph (SSC vs 7-AAD) and upon gating of 7-AAD unstained viable cells this gate was applied to a CD45-SSC dot plot graph (Figure 2).

Cell count and viability results for the CD34+ end products are summarized in Table 1.

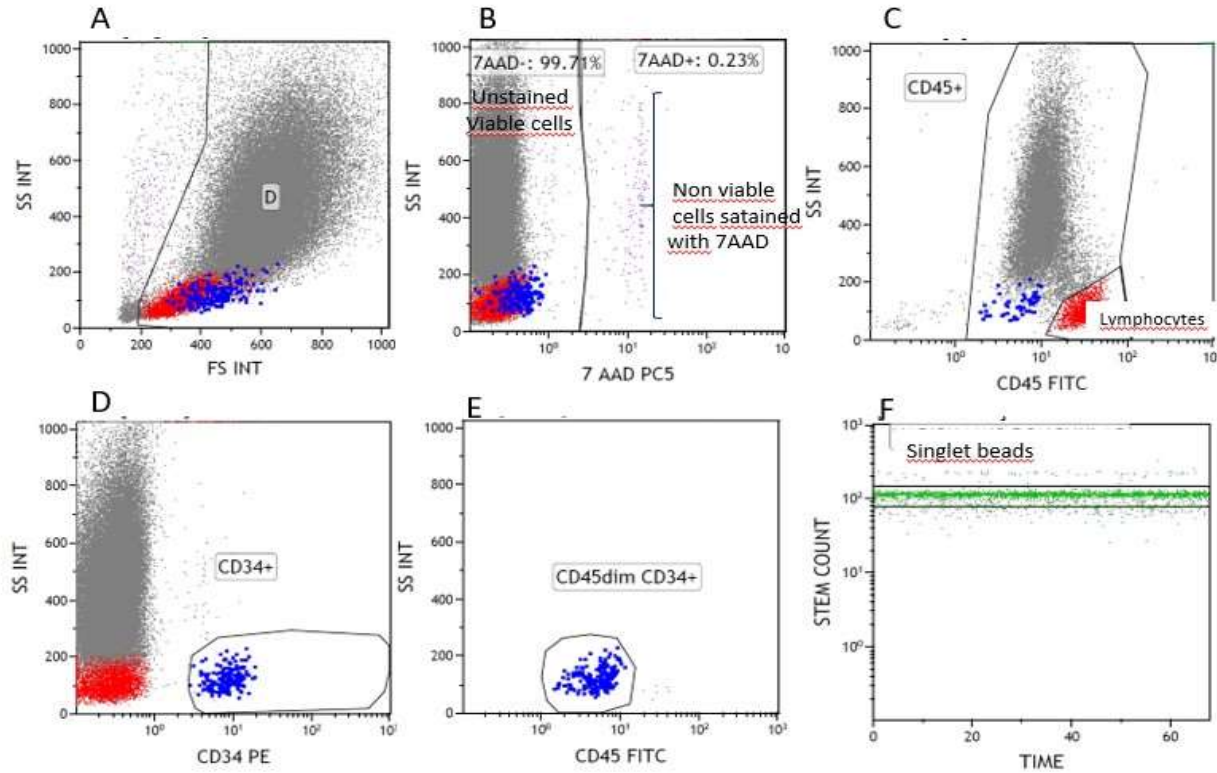
Mean values for cell count were estimated as  $3.44 \pm 1.22 \times 10^6/\text{ml}$  (range,  $2.48\text{-}5.71 \times 10^6/\text{ml}$ ) and  $4.14 \pm 1.94 \times 10^6/\text{ml}$  (range,  $1.77\text{-}7.43 \times 10^6/\text{ml}$ ) for the trypan blue exclusion and radio frequency tests, respectively. Additionally, viability mean values for the automated trypan blue exclusion and 7-AAD were  $93.38 \pm 6.09\%$  (range,  $79.00\text{-}98.00\%$ ) and  $99.49 \pm 0.60\%$  (range,  $98.40\text{-}100.00\%$ ), respectively.

#### **Discussion**

Automated techniques facilitate the work load of researchers by requiring less time for analysis and no need for complementary devices, and leave negligible effort. There is debate about the efficiency between the current automated cell count techniques. This issue has



**Figure 1.** An example of three consecutive cell count and viability results obtained by the use of the automated cell counter.



**Figure 2.** Before starting the enrichment of the hematopoietic stem cells; CD34<sup>+</sup> cell count was performed using the ISHAGE protocol: A: The artifacts were eliminated from the leukocytes, B: The viable cells that were unstained were selected, C: CD45<sup>+</sup> cells were selected from viable cells, D: CD34<sup>+</sup> cells among viable CD45<sup>+</sup> cells were marked, E: CD45<sup>dim+</sup> cells were selected from CD45<sup>+</sup> and CD34<sup>+</sup> viable cells and upon checking for particles smaller than lymphocytes (unseen) the actual viable CD34<sup>+</sup> cell numbers were detected. As seen in F, non-adhered beads, pipetted into the same tube, were selected and used for the absolute count of CD34<sup>+</sup> cells using the single platform analysis.

**Table 1.** Cell count and viability results by means of the two automated cell count techniques, and by means of an automated cell counter and flow cytometric evaluation by 7-AAD.

	Cell Count		Viability	
	Automated trypan blue exclusion (x10 <sup>6</sup> /ml)	Automated radio frequency (x10 <sup>6</sup> /ml)	Automated trypan blue exclusion (%)	7-AAD (%)
1	2.80	3.46	95.00	99.00
2	2.63	3.00	98.00	99.50
3	2.60	3.50	92.00	99.90
4	4.87	4.97	94.00	100.00
5	3.68	7.43	96.00	99.10
6	2.71	1.77	79.00	98.40
7	2.48	2.68	96.00	100.00
8	5.71	6.34	97.00	100.00

been addressed in a number of studies. The use of such techniques force the researchers to analyze the reliability of the automated systems. A number of previous studies have compared the various cell count and viability assessment methods [5,6,9-11,17].

Several studies have focused on cell count and viability of human cells. Leong et al. [9] used the flow cytometry technique for counting CD34+-selected hematopoietic stem cells and a manual trypan blue exclusion technique via the Neubauer chamber for viability assessment. We analyzed the same kind of cells and used the 7-AAD for viability assessment. However, while they preferred the manual technique for trypan blue exclusion, we used an automated cell counter. In the study of Leong et al. [9], the CD34+-selected products showed a median viability of 98%. (range 92 - 99%). We found only one published study which specifically addresses comparison of various techniques for cell count. Nevertheless, this study was not performed on human stem cells [11]. Until today, only two reports have studied the techniques on human stem cells on a viability perspective [6,17].

With the trypan blue exclusion method, Humpe et al. [17] detected the mean viability as 95.8% (range, 72.6%-98.7%) for 8 patients, with slight similarity to our automated viability results, 93.38±6.09% (range, 79.00-98.00%). Like us, Varan et al. [6] studied the comparison of viability results belonging to hematopoietic progenitor cells in 20 samples by trypan blue uptake and measurement of 7-AAD staining by flow cytometry. However, no remarks were made on cell count [6]. The median viability obtained by the 7-AAD was 78±16%, much lower from our 7-AAD results, of 99.49±0.60% (range, 98.40-100.00%). The Intraclass Correlation Coefficient (ICC) between the trypan blue and 7-AAD methods was found as

0.47 ( $p > 0.05$ ) in the study Varan et al. [6], and no statistically significant concordance was detected. In our study, no significant correlation was detected between viability results obtained by the two different techniques, the automated cell counter and the 7-AAD. Reich-Slotky et al. [10] determined the number of CD34+ cells by flow cytometry and the viability by trypan blue uptake and by the measurement of 7-AAD staining using flow cytometry. The average viability was 98.8% with trypan blue exclusion, and 97.0% with 7-AAD [10]. Our study suggested mean values of viability obtained by the automated cell counter as 93.38±6.09% (range, 79.00-98.00%) and of 7-AAD as 99.49±0.60% (range, 98.40-100.00%). Such values were respectively lower and higher than that of Reich-Slotky et al. [10]. These findings are controversial and thus make it questionable as to which method would be more reliable.

Previously, there have been different results comparable with ours obtained from mammalian cell types of non-human origin. Similar with our study, Camacho-Fernández et al. [11] performed cell counting by comparing manual and automated techniques in isolated eggplant microspore cultures. This study is significant as it has compared several techniques for cell count. In our study, when the two different automated cell counters were compared by means of cell count results, a significant correlation value of 0.72 was observed, in concordance with that of Camacho-Fernández et al. [11]. On the other hand, Kwizera et al. [5] counted *Cryptococcus* yeast cells in cerebrospinal fluid culture by trypan blue staining and rapidly quantified viable cells with an automated cell counter. The study of Kwizera et al. [5] is the first article practicing the comparison of different automated cell counting methods on human

stem cells and mainly focusing on the validation of the repeatability of results.

Although human donor-based, cellular therapies are classified as human medicinal products, they differ from the conventional medicines in the sense that each batch release is equivalent to one donor per manufacture. In this manner, it is not so easy to reach high sample sizes as it would be for serial manufacturing. Additionally, one end user, a patient, has to be matched with the appropriate donor in order to start manufacturing a cellular therapy product. This does not always happen in a serial manner, which also contributes to the bottleneck of reaching high sample sizes. In the setting of our study, the current sample size for hematopoietic stem cell manufacturing is 8. We plan further studies to enhance this sample size and continue our studies in depth.

### **Conclusion**

In conclusion, our study has responded to two questions at the same time: whether the results of both automated techniques for cell count correspond with each other, and whether the results of the automated viability assessment conform those of the 7-AAD technique when manufacturing cellular therapy products intended for clinical use. Even though we have the opportunity to use the hemocytometer in our laboratory setting, the automated cell counter that make use of trypan blue exclusion gives the cell count results in concordance within the range of the expectations of the Quality Management System (QMS) of our Tissue and Cell Manufacturing Center. However, for viability results, the 7-AAD technique, which is already validated at our premises, might be more accurate when products are intended for clinical use.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Ethical statement:** The study was approved by the Turkish Ministry of Health Turkish Medicines and Medical Devices Agency, with the approval number 2014/2 for manufacturing of human medical products.

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## Cervical lymphadenopathy in tularemia: the role of diffusion-weighted magnetic resonance imaging in differentiating lymphadenopathies due to metastatic tumors

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### ABSTRACT

**Aim:** To evaluate the role of diffusion-weighted magnetic resonance imaging (DW-MRI) in differentiating enlarged cervical lymph nodes due to tularemia and metastatic tumors.

**Methods:** We evaluated 59 patients with cervical lymphadenopathy (LAP) (32 patients with tularemia, 27 patients with metastatic tumors), retrospectively. We analyzed contrast enhancement patterns of LAP in postcontrast fat sat T1WI. We evaluated T2, DWI, and ADC signals of LAP in a 5-point scale system. Moreover, the mean ADC values of solid and necrotic LAP in both groups were quantitatively measured and compared statistically. Receiver operating characteristic curves of quantitative ADC values were obtained to determine the diagnostic performance.

**Results:** There was no difference between solid and necrotic LAP enhancement patterns in two groups. Solid LAP and peripheral parts of necrotic LAP showed diffusion restriction, whereas central parts necrotic LAP had high ADC and low DWI signal in both tularemia and metastatic groups. Signal characteristics were similar in two groups. In solid LAP, there was no significant difference between ADC values in two groups. In necrotic LAP, total, central, and peripheral quantitative ADC measurements were higher in the metastatic group than in the tularemia group.

**Conclusions:** Conventional MRI findings were not sufficient to differentiate metastatic LAP from tularemia. DW-MRI was not helpful in solid LAP; however, ADC values of metastatic necrotic LAP were significantly higher than tularemia. Microagglutination tests would be useful for differentiation; however, DW-MRI might also be useful for differentiation and may expedite the diagnosis.

**Keywords:** Cervical LAP, tularemia, metastatic tumors, necrotic LAP, MRI, DW-MRI.

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### Introduction

Tularaemia is a zoonotic bacterial infectious disease caused by a gram-negative

coccobacillus, *Francisella tularensis*. It is a rare pathogen for the urban population; however, it is more common in rural areas and a potential agent for bioterrorism. Diagnosis of tularaemia is difficult due to; its rarity and inconsistency of clinical findings, fastidious nature that causes weak growth in standard culture media, and confusing histopathological appearance mimicking tuberculosis [1-4].

The disease is commonly found in the soft tissue of the head and neck region. Imaging is mainly used to show the lesion and its extension. Since tularaemia is usually presented as neck masses with confusing clinical findings, differentiating them from malign lymphadenopathies is essential. Therefore, in addition to conventional contrast-enhanced MRI, diffusion-weighted magnetic resonance imaging (DW-MRI) findings could be helpful for differential diagnosis. DW-MRI is a recognized and reliable tool to evaluate and characterize lymph nodes. It is based on diffusion properties of water protons in biologic tissues, including the diffusion of molecules in extracellular or intracellular spaces as well as in cell membranes. There were numerous reports of DW-MRI characterization of malign, and benign lymph nodes might be completed without the need for invasive procedures [5-8]. The aim of this study is to evaluate the DW-MRI features of lymph node involvement due to tularemia and metastatic tumors in the head and neck region and to show the similarities and differences between them.

### **Materials and Methods**

We obtained medical records of the patients who had tularaemia diagnosis serologically in our institution between June 2009 and June 2019. All patients underwent neck ultrasonography (US) and neck magnetic resonance imaging (MRI). Ethics committee approval was obtained and informed consent was waived by ethics committee because of the retrospective study design. Informed consent was waived because of the retrospective design and institutional ethical board approved our study (Decision No: 2020/28 Date: 18.02.2020) Patients were divided into two main groups as tularemia and metastasis. Both groups were divided into two subgroups as solid and

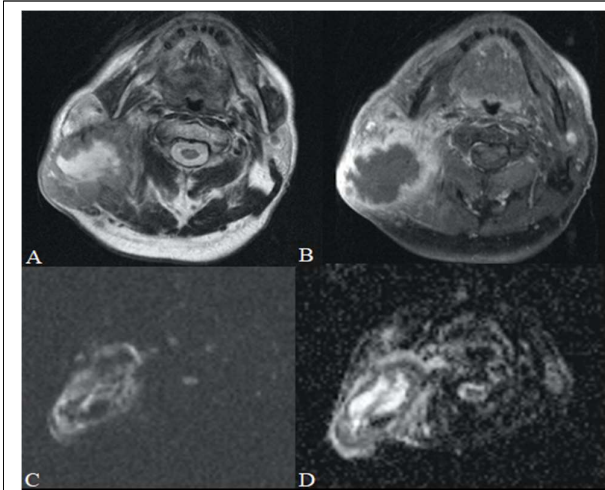
necrotic. Serological diagnosis of tularaemia was made with the existence of specific antibody titers of  $\geq 1:160$  or with at least a fourfold increase of antibody titer in two serum samples taken two weeks apart in patients with clinical findings and symptoms which is compatible for tularaemia [9]. Thirty-nine patients were meeting these criteria. We excluded seven patients because they had undergone biopsy or surgical drainage before the MRI examination.

Patients in the metastatic group, who had a histopathological diagnosis of metastasis and neck US and neck MRI in our hospital between June 2009 and June 2019, were obtained. Twenty-nine patients were meeting these criteria. We excluded two patients from the study. One of the patients had insufficient biopsy material and discontinuation of the follow-up. Other patient presented with caseified granulomatous lymphadenitis.

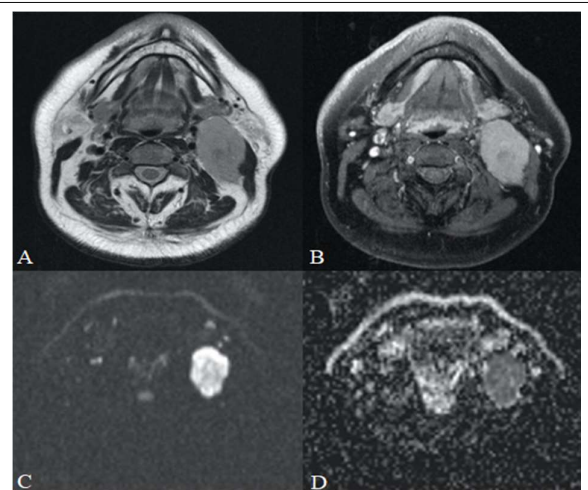
All patients underwent MRI examination with a 1.5 Tesla MRI machine (Symphony; Siemens Medical Systems, Erlangen, Germany). A head and neck surface coil was used. MR images of patients were uploaded to a workstation (syngoMMWP VE25A, Siemens AG, Berlin, and Munich, Germany) for evaluations and measurements. Neck US images were not in PACS; therefore, we used US reports for comparisons.

### **MRI technique**

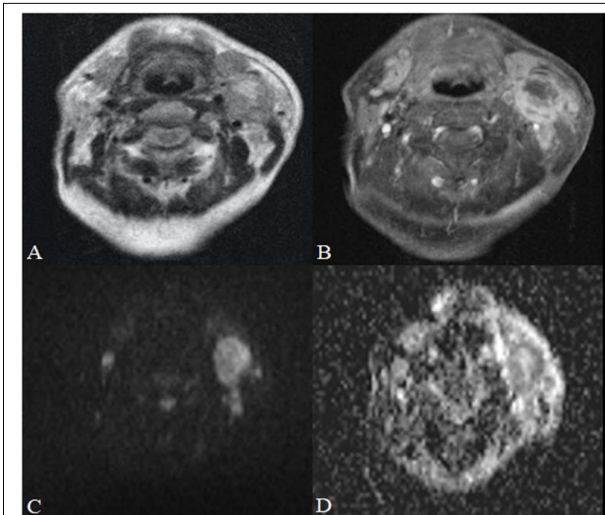
T1-weighted images (TR/TE of 800/20 ms) and T2-weighted fast spin-echo images (TR/TE of 6.100/85 ms) were obtained from all patients. For MR images, the section thickness was 5 mm, interslice gap was 1–2 mm, field of view (FOV) was 25–30 cm, and the acquisition matrix was 256x224. For diffusion-weighted magnetic resonance imaging (DW-MRI), a multislice, single-shot, spin-echo, echo-planar imaging sequence was used. The section



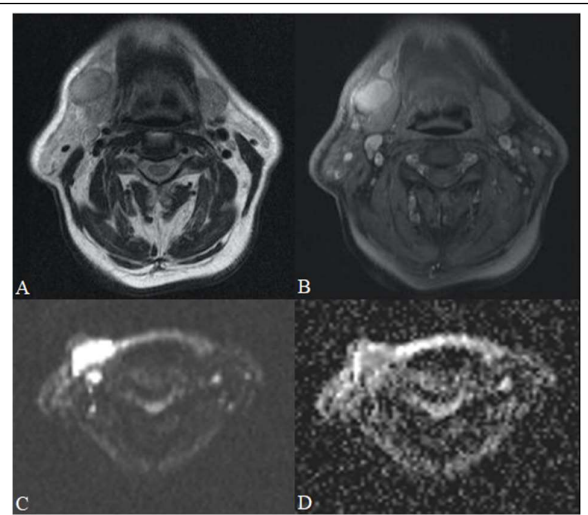
**Figure 1.** Metastatic necrotic LAP on the right side of the neck. (A) Central hyperintensity on Axial T2 weighted image. (B) LAP shows peripheral enhancement on T1 weighted image after contrast administration. (C) Axial DW-MRI of the necrotic LAP shows slight hypointensity on the central part and hyperintensity of the peripheral part of the lesion. (D) On the axial ADC image, necrotic LAP shows hyperintensity on the central part.



**Figure 2.** Metastatic solid LAP on the left side of the neck. (A) LAP shows intermediate signal on Axial T2 weighted image, (B) homogenous enhancement on T1 weighted image after contrast administration, and (C and D) restricted diffusion on DW-MRI.



**Figure 3.** Necrotic LAP in tularemia on the left side of the neck. (A) Central hyperintensity on Axial T2 weighted image, (B) peripheral enhancement on postcontrast T1 weighted image. (C) Axial DWI of the necrotic LAP shows slight hypointensity on the central part of the lesion in comparison to hyperintensity of the peripheral part and (D) hyperintensity on the central part on axial ADC image.



**Figure 4.** Solid LAP in tularemia on the right side of the neck. (A) LAP shows a slightly high signal on Axial T2 weighted image, (B) homogenous enhancement on T1 weighted image after contrast administration, and (C and D) restricted diffusion on DW-MRI.



thickness was 5 mm, TR:0.300 ms, TE: 70 ms, FOV read 400 mm, and FOV phase 66.7%. DW-MRI was obtained with a diffusion factor  $b$  of 0, 400, and 800  $s/mm^2$  with ADC maps in all patients.

### **Image interpretation**

Images and information of a total of 59 patients, 32 in the tularaemia group and 27 in the metastasis group, were scanned from the hospital information system. MRI and DW-MRI were evaluated by two radiologists who had one year and ten years of experience in radiology with a consensus reading. Image interpretation was blinded to clinical information.

We used the largest lymphadenopathies (LAP) in the evaluation. Lymph nodes that are larger than 1 cm in the smallest diameter and without cystic or necrotic component on US reports, with high DW-MRI and low ADC signal on MRI, were defined as solid LAP. Necrotic LAP were considered the lesions which were defined as cavitory lesions on US reports and had low DW-MRI and high ADC signal on MRI (Figure 1-4). In homogenously enlarged lymph nodes (solid LAP), a region of interest (ROI) was placed inside the margins of the lesion to measure ADC value. When there was heterogeneity in signal intensity of the lesion, a larger ROI for whole lesion and two smaller ROIs were placed within both cystic-necrotic areas and areas which showed enhancement after gadolinium administration. Mean ADC values of regions with high and low ADC signals were calculated separately.

We made minimal ROI size determination based on parameters suggested before [10]. Minimal ROI size was equal or larger than the voxel size to prevent partial volume effect would not produce unreliable measurements. We calculated the voxel size of each DWI-sequence separately using FOV, matrix size,

and slice thickness parameters. ROI size, which is smaller than this calculated voxel size, was not applied.

The signal intensities of the lesions on T2, DW-MRI and ADC map images were visually compared to the adjacent muscle using a 5-point scale system: 1: hypointense, 2: moderately hypointense, 3: isointense, 4: moderately hyperintense, 5: significantly hyperintense. Because the DW-MRI sequence had a low spatial resolution, borders of ROI were defined with the guidance of conventional MRI. We made the quantitative ADC measurements on ADC map images. Mean ADC values, which were measured by the same two radiologists, and the differences were decided with a consensus.

### **Statistical analysis**

Quantitative variables are summarized with mean  $\pm$  standard deviation values. Qualitative variables were compared with the Chi<sup>2</sup> test between the two groups. Because of continuous variables did not provide the assumption of normality, non-parametric Mann Whitney U test was used to compare the groups. We performed data analysis in SPSS 25.0 program (SPSS Inc., Chicago, Illinois, USA) and interpreted statistical tests at  $p < 0.05$  significance level.

### **Results**

In the tularaemia group (n: 32), there were 18(56%) solid, 14(44%) necrotic LAP. In the metastatic group (n: 27), there were 18(67%) solid and 9(33%) necrotic LAP. In the tularaemia group, 20(62%) of the patients were male, 12(38%) of the patients were female. In the metastatic group, 20(74%) of the patients were male, 7(26%) of the patients were female (Table 1). The mean age and age range of the patients in the tularaemia and metastatic groups were shown in table 2.

**Table 1.** Demographic information (sex).

Parameters	Metastatic	Tularemia	Total
Male	20	20	40
Female	7	12	19
Total	27	32	59

**Table 2.** Demographic information (age).

	Metastatic (27)		Tularemia (32)		Total (59)
	Solid (18)	Necrotic (9)	Solid (18)	Necrotic (14)	
Mean age	54,3	59	52,5	51,7	53,9
Age range	24-76	25-78	22-71	20-68	20-78

All necrotic LAP, regardless of tularaemia or metastatic group, showed peripheral contrast enhancement. In patients with solid LAP, we found homogeneous contrast involvement in 13 patients in the tularaemia group, 11 patients in the metastasis group, 5 patients in the tularaemia group, and 7 patients in the metastasis group. There was no significant difference between solid and necrotic LAP enhancement patterns in tularaemia and metastasis groups ( $p > 0.05$ ).

T2 signal was high in all lesions, mostly in the central part of necrotic LAP. Solid LAP and peripheral parts of necrotic LAP showed high DWI and low ADC signal, whereas DWI and ADC signals of central parts of necrotic LAP were the opposite (Table 3-5).

**Table 3.** Qualitative mean MRI scores of tularaemia and metastatic solid lymphadenopathies.

Sequence	Tularaemia	Metastasis	<i>p</i>
T2WI	4.1	4	0.923
DW-MRI	4.8	4.66	0.865
ADC	1	1.22	0.629

There was no significant difference between tularaemia and metastasis groups in terms of qualitative signal characteristics.

**Table 4.** Qualitative mean MRI scores of tularaemia and metastatic necrotic lymphadenopathies.

	Central			Peripheral		
	T2WI	DWI	ADC	T2WI	DWI	ADC
Tularaemia	5.0	2.71	5.0	3.93	4.54	2.3
Metastasis	5.0	2.27	5.0	3.72	4.81	2.18
<i>p</i>	1.0	0.226	1.0	0.563	0.061	0.857

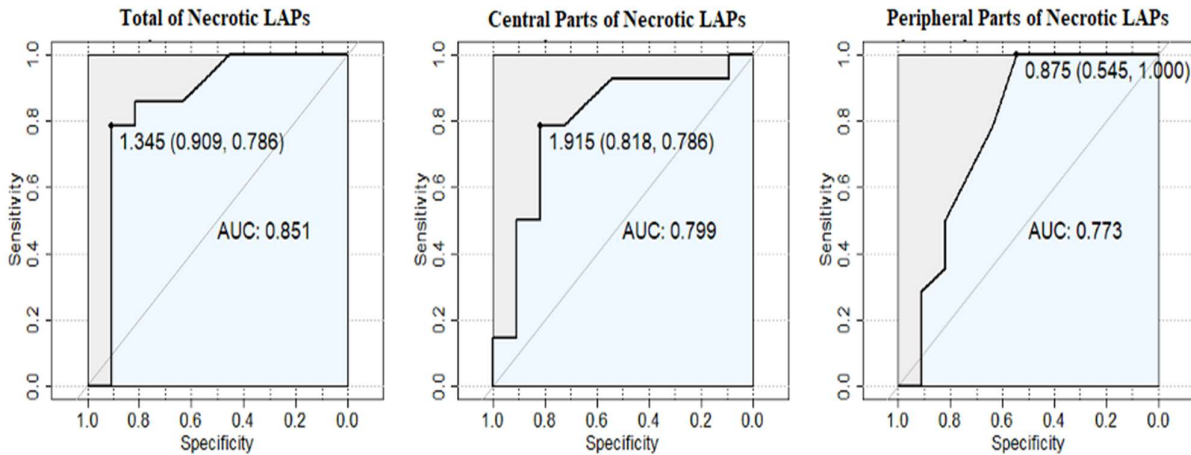
In solid LAP patients, there was no significant difference between ADC measurements in tularaemia and metastasis groups. In necrotic LAP patients, total, central and peripheral ADC measurements were significantly higher in the metastatic group than in the tularaemia group. According to the ROC analysis, ADC cut-off values were found to be  $1.915 \times 10^{-3} \text{ mm}^2/\text{s}$  (Sensitivity: 81.8%, Specificity: 78.6%, AUC: 0.799,  $p=0.012$ ) for central portions of necrotic LAP,  $0.815 \times 10^{-3} \text{ mm}^2/\text{s}$  (Sensitivity: 81.8%, Specificity: 50%, AUC: 0.773,  $p=0.021$ ) for peripheral portions of necrotic LAP and  $1.345 \times 10^{-3} \text{ mm}^2/\text{s}$  (Sensitivity: 90.9%, Specificity: 78.6%, AUC: 0.851,  $p=0.003$ ) for total part of necrotic LAP to differentiate metastatic and tularaemia groups in necrotic LAP (Figure 5).

### Discussion

We found that necrotic LAP had significantly higher ADC values in the metastatic group in comparison to tularaemia. Differentiation of malign LAP from tularaemia cases with head and neck involvement in MRI or DWI might not be possible and could cause a diagnostic dilemma. In this case, if the LAP is necrotic, ADC values have diagnostic value for differentiation of metastasis.

**Table 5.** Quantitative mean ADC values of tularaemia and metastatic lymphadenopathies.

	Solid ( $10^{-3}$ mm <sup>2</sup> /s)	Necrotic		
		Total ( $10^{-3}$ mm <sup>2</sup> /s)	Central ( $10^{-3}$ mm <sup>2</sup> /s)	Peripheral ( $10^{-3}$ mm <sup>2</sup> /s)
<b>Tularaemia</b>	0.74 ± 0.05	1.21±0.21	1.76±0.44	0.80 ±0.05
<b>Metastasis</b>	0.76 ± 0.12	1.67±0.35	2.32±0.49	0.92± 0.20
<b>P</b>	0.372	0.002*	0.011*	0.021*



**Figure 5.** ROC analysis of ADC values according to total, central, and peripheral parts of necrotic LAP to discriminate tularaemia from metastasis.

In our study, both tularaemia and malignant solid LAP had low mean ADC values and were hypointense on ADC and had similar mean ADC values. In previous studies, the mean ADC values in metastatic LAP were reported as  $0.59 \pm 0.27 \times 10^{-3}$  mm<sup>2</sup>/s,  $0.78 \pm 0.09 \times 10^{-3}$  mm<sup>2</sup>/s,  $0.85 \pm 0.27 \times 10^{-3}$  mm<sup>2</sup>/s [12-14]. Furthermore, there are several reported cases of LAP with granulomatous reaction such as sarcoidosis and cat scratch disease, that present low ADC values resembling malignancy [10, 15, 16]. The reason for restricted diffusion in tularaemia might be the ability of the pathogen to impair phagocyte function, and thus survive in the infected cells [17].

We found that ADC and T2 signals were high in the central part of the necrotic LAP in both

groups. This situation was also similar for metastatic necrotic LAP and contrary to necrotic lymphadenitis in the study of Koç et al. [18]. However, in qualitative evaluation, mean ADC values of central parts and the total of necrotic LAP were significantly higher in the metastatic group than the tularaemia group. When thresholds of mean ADC values were determined as  $1.915 \times 10^{-3}$  mm<sup>2</sup>/s for the central part and  $1.345 \times 10^{-3}$  mm<sup>2</sup>/s for the total of necrotic LAP, differentiation is probable with high sensitivity and specificity. Since necrosis is a factor that increases diffusion, ADC values of the peripheral solid parts of the necrotic LAP, which are viable, were similar to the ADC values of LAP without necrosis. However, diffusion increases in the necrotic parts,

regardless of emerging due to tularaemia or malignant causes [19]. In our study, central, peripheral, and total ADC values of necrotic LAP were significantly higher ( $p=0.011$ ) in the metastatic group than in the tularaemia group by the quantitative ADC evaluation. Koç et al. reported that ADC values of tumoral necrotic lymph nodes were significantly higher than those of infective necrotic lymph nodes, and they attributed that the movement of water molecules was freer than infective necrosis with high protein content, as a reason of this. Whereas Koç et al. reported that infective necrotic lymph nodes were hyperintense in DWI, DWI was hypointense in the necrotic parts of LAP in tularaemia group in our study [18]. However, ADC values of tumoral necrotic LAP were significantly higher than necrotic LAP in tularaemia. This condition is thought to be related to the intense content with necrosis in tularaemia, and it can be used as a parameter to differentiate tularaemia-metastasis. However, considering that this situation may be due to the low number of our study population, studies with a larger population are needed on this subject.

Although the mean ADC values of the peripheral part of the necrotic LAP were also significantly higher in metastatic groups than the peripheral group, specificity was lower than the central parts or total of the necrotic LAP. This condition might be due to the effect of large central necrotic part of the LAP on the thin peripheral part in which ROI placed because of the low resolution of DWI.

There were certain limitations to this study. First, since a limited patient population might restrain the results, they should be confirmed on a larger scale. Second, US or fine-needle aspiration could not be performed by us, and we obtained the reports of these procedures from the archives. Third, we evaluate images with a

consensus of two observers, so we did not assess interobserver variability.

Both tularaemia and metastases cause solid and necrotic LAP in the neck. Conventional MRI findings do not provide sufficient information to differentiate these lesions. There was no significant difference in tularaemia and tumour in terms of signal characteristics in solid LAP. Since tularaemia is an infectious disease, clinical findings might be considered useful in differentiation; however, confusing clinical presentation is not uncommon in tularaemia. Microagglutination test will be useful in the evaluation of these lesions. The microagglutination test might be used in necrotic LAP; however, despite tularaemia and tumour signal characteristics are similar, higher ADC values might favour the diagnosis of tumour in necrotic LAP than tularaemia and might be useful for differentiation and may expedite the diagnosis.

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## Cholelithiasis and gastrointestinal cancer: Is there a relationship that increases the risk of developing cancer?

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### ABSTRACT

**Aim:** To investigate the co-existence of cholelithiasis in patients with gastrointestinal (GI) cancer both in preoperative and postoperative periods.

**Methods:** We retrospectively analyzed the data of patients who underwent GI tract cancer surgery in the general surgery clinic of a university hospital between January 2013 and December 2019 for the presence of 'cholelithiasis' in the preoperative and postoperative periods. Age, gender, tumor type and localization and presence of the cholelithiasis in the patients were determined. In addition, the cases were divided into two as upper GI tract and lower GI tract according to tumor location and the relationship with cholelithiasis was evaluated.

**Results:** A total of 680 GI cancer patients were included in the study. Localization of GI cancers were; colon in 211 cases (31%), rectum in 195 cases (28.7%), gastric in 187 cases (27.5%), periampullary region in 55 cases (8.1%), and small intestine in 32 cases (4.7%). In the preoperative period, 69 (10.1%) patients were associated with cholelithiasis. Thirty-one (5.1%) patients had accompanying cholelithiasis in the postoperative period. Coexistence of cholelithiasis according to cancer location was not statistically significant in the preoperative and postoperative periods.

**Conclusions:** Our available data make it difficult to distinguish the roles of cholelithiasis on gastrointestinal cancers, because no statistically causal relationship was found between cholelithiasis and gastrointestinal cancers. However, the role of asymptomatic and symptomatic stones, which may or may not require cholecystectomy, in the development of GI tract cancers should not be ignored.

**Keywords:** Cholelithiasis, gallbladder diseases, cholecystectomy, gastrointestinal tract cancer.

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### Introduction

Cholelithiasis or gallstone disease is a common problem of the digestive system. It has an 11%

to 36% prevalence in different series [1]. It is more common in females and after 40 years of age, and the risk of gallbladder stone increase 2 folds in the relatives of the patients with gallbladder stone [2]. Moreover, a history of gastrointestinal surgery also increases the risk of gallbladder stone disease [3]. The most important task of the gallbladder is to store the hepatic bile necessary for digestion in the

gastrointestinal (GI) tract in adequate quantity, and discharge it into the duodenum when required. Cancers occurring in the gastrointestinal tract cause major disruptions in the physiological process depending on their localization. The problem grows even more when patients face cancer and cholelithiasis. Associations between cholecystectomy and colorectal cancer have been demonstrated previously [4,5]. On the other hand, association between small intestinal carcinogenesis and cholecystectomy is less clear and since the disease is rare data about this subject only based on 4 small studies [6]. Consequently, cancers of the GI tract have been focused on since there is a possible relation between cholelithiasis and cancer risk. In our study, we aimed to investigate the association of cholelithiasis in the preoperative and postoperative period of patients that undergone surgery for GI tract cancer.

### Materials and Methods

We retrospectively analyzed the data of patients who underwent GI cancer surgery between January 2013 and December 2019 in General Surgery Department of a University Hospital. This study was approved by the institutional directorate and ethical board (No/date: 103548508/14.10.2019). Patients who underwent cholecystectomy without a diagnosis of GI cancer were excluded from the study. The coexistence of cholelithiasis in the preoperative period and postoperative period was investigated. Patients diagnosed with cholelithiasis in the preoperative period were excluded from the group in the postoperative period. Age, gender and tumor localization of the patients were determined. The association of tumor localization and cholelithiasis was determined in the preoperative and postoperative periods. According to tumor

localization, the cases were divided into two as; upper GI and lower GI (distal of the treitz ligament).

### Statistical analyses

The demographic parameters and pathological results of all patients were recorded and statistically analyzed by SPSS software (SPSS 15.0 for Windows, IBM Inc., Chicago, IL, USA). The non-homogenously distributed quantitative variables in study groups were compared by the Mann-Whitney U Test and expressed as median (IQR). The qualitative variables were analyzed by the Chi-Square Test and expressed as n (%). Multivariate analysis was performed in comparison of cholelithiasis cancer association in preoperative and postoperative period. A  $p$  value less than 0.05 was considered statistically significant.

### Results

The study included 680 GI cancer patients. Cholelithiasis was detected in 100 (14.7%) patients. The median ages the patients with and without cholelithiasis were 66 (35-89) and 65 (17-92), respectively ( $p = 0.74$ ). A total of 407 (59.9%) of the patients were male and 41 (6%) of them had accompanying cholelithiasis in the preoperative period; and 273 (40.1%) of the patients were female and 28 (4.1%) of them had accompanying cholelithiasis in the preoperative period. The coexistence of cholelithiasis between male and female genders in the preoperative period was not statistically significant ( $p = 0.93$ ). Localization of GI cancers were as follows; colon 211 (31%), rectum 195 (28.7%), stomach 187 (27.5%), periampullary region 55 (8.1%) and small intestine 32 (4.7%). In the preoperative period, 69 (10.1%) patients had accompanying cholelithiasis, and their distribution was as follows; 22 (3.2%) colon, 20 (2.9%) rectum, 18 (2.6%) gastric, 6 (0.9%) periampullary, and 3



(0.4%) small bowel cancers. According to cancer localization, cholelithiasis coexistence was not statistically significant in the preoperative period ( $p = 0.94$ ) (Table 1).

**Table 1.** Association of the preoperative cancer and cholelithiasis.

Parameters	Preoperative cholelithiasis		p
	Yes (N, %)	None (N, %)	
Age (years)	66/18	65 / 82	0.74
Gender			0.93
Male	41/6	366/54	
Female	28/4.1	245/36	
Location			0.94
Colon	22/3.2	189/28	
Rectum	20/2.9	175/26	
Stomach	18/2.6	169/25	
Periampullary	6/0.9	49/7.2	
Small intestine	3/0.4	29/4.2	
Upper GI tract	24/3.5	218/32	
Lower GI tract	45/6.6	393/58	0.88

For the postoperative period, 611 patients were included in the study. A total of 366 patients were male, 18 had cholelithiasis, and 13 of 245 female had cholelithiasis. The association with cholelithiasis in the postoperative period was not statistically significant ( $p = 0.48$ ). In the postoperative period, 31 (5.1%) patients were associated with cholelithiasis, and their distribution by localization were as follows; 13 (2.2%) colon cancer, 8 (1.3%) rectum cancer, 8 (1.3%) stomach cancer, 2 (0.3%) periampullary region cancer. The association of cholelithiasis in the postoperative period with localization was not statistically significant ( $p = 0.35$ ) (Table 2).

In the preoperative period, 242 (35.6%) upper GI cancer patients (3.5%) had cholelithiasis association in 24 of 438 (64.4%) patients with

**Table 2.** Association of the postoperative cancer and cholelithiasis.

Parameters	Postoperative cholelithiasis		p
	Yes (N, %)	None (N, %)	
Age(years)	66/5	65/95	0.74
Gender			0.48
Male	18/2.9	348/57	
Female	13/2.1	232/38	
Location			0.35
Colon	13/2.2	176/30	
Rectum	8/1.3	167/28	
Stomach	8/1.3	161/28	
Periampullary	2/0.3	47/9	
Small intestine	0/0	29/5	
Upper GI	10/1.6	208/34	
Lower GI	21/3.5	372/60	0.68

**Table 3.** Comparison of patients with cholelithiasis in the preoperative and postoperative period.

Parameters	Preop. Chl (N, %)	Postop. Chl (N, %)	p
Gender			0.97
Male (n= 407)	41/6	18/3	
Female (n= 273)	28/3	13/2	
Location			0.92
Colon (n=211)	22/3.2	13/2.2	
Rectum (n= 195)	20/2.9	8/1.3	
Stomach (n= 187)	18/2.6	8/1.3	
Periampullary (n= 55)	6/0.9	2/0.3	
Small intestine (n= 32)	3/0.4	0/0	
Upper GI (n= 242)	24/3.5	10/1.6	0.91
Lower GI (n= 438)	45/6.6	21/3.5	

Preop. Chl: preoperative cholelithiasis. Postop. Chl: postoperative cholelithiasis.

lower GI cancer, and this rate was not statistically significant ( $p = 0.88$ ). In the

postoperative period, cholelithiasis coexistence was detected in 10 (1.6%) of 218 (35.7%) upper GI cancer patients and in 21 (3.5%) of 393 (64.3%) lower GI cancer patients, and this rate was not statistically significant ( $p = 0.68$ ). Only 1 of the patients who underwent cholecystectomy had bile leak that improved with medical treatment.

In multivariate analysis, the association of cholelithiasis and cancer in the preoperative and postoperative period was not statistically significant ( $p = 0.91$ ) (Table 3).

### **Discussion**

In present study, we showed that coexistence of cholelithiasis was not statistically significant in our patients with gastrointestinal cancer before and after the surgical procedures. However, the rate of cholelithiasis appears higher in the preoperative and postoperative period in patients with colon, rectum and gastric cancer. The treatment process in the gastrointestinal system cancers is difficult and takes a long time. Treatments that vary according to the stage of cancer can be quite complicated. Adding cholelithiasis to this situation before or after cancer surgery forces clinicians and the patient. There are studies showing that whether cholelithiasis is associated with GI cancers or not. Although the relation between cholecystectomy and colorectal cancer was considered in many studies, the results were equivocal; most of the case-control studies showed a positive relation, but only the two largest cohort studies showed significantly increased risks, which were restricted to women and to the proximal part of the colon [7-9]. In studies conducted in the US and Norway, gallstones were not found to be related to colorectal cancer [10,11]; whereas, in other studies conducted in the US and Japan, cholelithiasis was shown to be positively

related to CRN [12,13]. In our study, the correlation of lower GI cancers in the preoperative and postoperative period was found as high as 6.6% -3.5%.

Biliary constituents were reported to be genotoxic and to cause local cellular damage which consequently increase mitotic activity of damaged tissue [14]. Therefore, most of the adenocarcinomas of the small intestine occur near the ampulla of Vater in the duodenum, which supports the hypothesis that bile was a carcinogen for the small intestinal mucosa especially in proximity with the site where bile is excreted [14]. Tavani et al suggested that there was a positive association between cholelithiasis and the risk of small intestinal cancer and provide quantitative estimates of the overall association [6]. However, in our study, the rate of cholelithiasis was very low in patients with small bowel cancer.

Increased release of bile acids into the duodenum during fasting is believed to be responsible of increased risk of colorectal cancer following gallstone disease and cholecystectomy [15,16]. The concentration of bile acids increase also by increased enterohepatic circulation [17]. Deoxycholic, a secondary bile acid which is carcinogenic especially with increasing concentrations, is increased by bacterial deconjugation and 7 $\alpha$  dehydroxylation of primary bile acids in the proximal colon [18]. A relationship was found more often between colorectal cancer and cholelithiasis, that with cholecystectomy reported by Novell et al [19]. No significant differences in gallstone and bile composition between colon cancer patients with concomitant gallstones and control groups have been found by Gafa et al. [20]. However, a higher incidence of bile bacteria (35.7%) was observed in cancer patients with gallstones. right colon cancer patients who had pigment

stones in 75% of the cases reported to have more frequent bile bacteria. The results seem to evidence peculiarities in patients with a cancer of right colon [20]. In our study, concomitant cholelithiasis was 32% preoperatively and 42% postoperatively in patients with colon cancer. The incidence of gallstone formation has been regarded as one of the most common complications after gastrectomy [21-23]. The underlying pathophysiology of this phenomenon for this postoperative disease has included alterations in gallbladder motility, the release of cholecystokinin (CCK), and gallbladder responses [24,25]. However, several studies have shown a higher rate of gallstone formation after gastrectomy [26]. The reported incident rate was usually around 10-25%, but rates as high as 47 and 60% had been cited in previous studies [21,26,27]. While the rate of cholelithiasis in the preoperative period was 21% in patients who are planned for obesity surgery, this rate reached 52% in the postoperative period [28]. Approximately 6% of patients undergoing upper GI surgery are expected to require cholecystectomy during follow-up [26]. In our study, the relationship between cholelithiasis in the preoperative and postoperative period was found at a rate of 3.5-1.6% in upper GI cancers. Cholelithiasis surgery has a specific morbidity and mortality burden. In the study of Cholegas, adding cholecystectomy to gastric cancer surgery did not significantly affect perioperative morbidity, mortality, and costs. However, one case (1.5%) of biliary leakage that was probably caused by prophylactic cholecystectomy was observed [29]. In our study, we found the rate of bile leakage as 0.15% in cholecystectomies that we performed with cancer surgery.

### **Conclusion**

In conclusions, the current data make it difficult to distinguish the roles of cholelithiasis on

gastrointestinal cancers, since the association of cholelithiasis and gastrointestinal cancers does not show statistical significance. Future research with larger population should focus on this subject to establish a causal link between cholelithiasis and gastrointestinal cancer. Therefore, efforts should be made to differentiate between the roles of asymptomatic and symptomatic stones, which may or may not require cholecystectomy. In addition, it is important that future studies adjust for major confounders especially studies exploring the risk of gastrointestinal cancer after exposure to gallstones.

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## The role of lymphocyte-monocyte ratio and platelet to lymphocyte ratio in predicting risk groups in gastrointestinal stromal tumors

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### ABSTRACT

**Aim:** Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract. Armed Forces Institute of Pathology (AFIP) criteria which is the basis of our study, is also known as Miettinen's criterion is used in classification of GIST. Lymphocyte-monocyte ratio (LMR), and platelet lymphocyte ratio (PLR) have been shown as novel markers in chronic systemic inflammatory response, therefore, we aimed to study LMR levels of the subjects with moderate to high risk GIST and to compare to those in the subjects with low or very low risk GIST.

**Methods:** Thirty GIST patients who underwent surgery were retrospectively evaluated. Patients were divided into two groups according to the AFIP risk scoring system: the first group (group 1) included very low and low risk patients and the second group (group 2) included moderate and high risk patients. Inflammatory indicators; LMR and PLR of the groups were compared.

**Results:** LMR value was higher in Group 1 ( $5.25 \pm 2.55$ ) than the LMR of group 2 ( $2.92 \pm 1.76$ ). PLR value was significantly lower in group 1 (139.68) compared to the PLR of group 2 (185.04).

**Conclusion:** We think that LMR is effective in identifying low and very low risk patients compared to AFIP. From this point of view, we suggest that LMR can identify high and medium risk patients by excluding low and very low risk patients and may be an independent risk factor in GIST scoring systems.

**Keywords:** Gastrointestinal stromal tumors, lymphocyte monocyte ratio, AFIP risk score.

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### Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract. The incidence of GIST is

14-20 per ml [1]. GIST are myenteric plexus small settled a kit positive cell population Cajall originate from cell neoplasms [2]. The main cause of GIST is presence of abnormal tyrosine protein kinase, which is known as CD117 that causes uncontrolled growth of gastrointestinal cells. GIST can occur anywhere in the gastrointestinal tract, such as, gastric (50-60%) and small bowel (30-35%), and less frequently in the colon and the rectum (5%). It is very rare (1%) in esophagus [3].

Although it may occur at any age, advanced age is a risk factor for GIST. The average age reported in previous studies is 60 years.

Different risk classification systems are used in GIST. Armed Forces Institute of Pathology (AFIP) criteria, which is the basis of our study, is also known as Miettinen's criterion is useful in risk stratification in GIST [1].

Virchow stated that there is a connection between cancer and inflammation and that lymphocytic infiltrate in the areas of chronic inflammation may constitute the origin of cancer [4]. Indeed, chronic inflammation has been documented in different types of cancer [5]. Since the systematic inflammatory response (SIR) indirectly reflects the host immune status, it probably reflects the prognosis of various malignancies, including gastrointestinal cancers. Alternatively, the lymphocyte-monocyte ratio (LMR), which is the ratio of monocyte count in peripheral blood to lymphocyte count, has been shown as a novel marker of chronic SIR. This is because monocytes, monocytes-derived macrophages and lymphocytes play primary role in chronic inflammation rather than acute inflammation [6]. Some studies have reported that LMR was a prognostic factor for disease-free survival and overall survival in colorectal cancer [7] and pancreatic cancer [8].

In this study, we investigated the effect of hemogram parameters obtained from preoperative blood count tests of subjects with GIST in predicting low and very low risk GIST patients according to AFIP scoring.

### **Materials and Methods**

Thirty patients with gastrointestinal stromal tumors who underwent surgery in the general surgery clinic of Bolu Abant Izzet Baysal University (BAIBU) Medical Faculty between 2012 and 2018 were retrospectively evaluated.

The study was approved by BAIBU, Clinical Research Ethics Committee, decision number 2016/369, dated 01/06/2016. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration, as revised in 2000.

When the files were retrospectively examined, hemogram values were included in the study seven days before the operation date. Preoperative hemogram parameters white blood cells (WBC), neutrophil, lymphocyte, monocyte, and platelet values were recorded. The ratio of neutrophil to lymphocyte ratio (NLR) by the ratio of neutrophil count to lymphocyte count, and lymphocyte monocyte ratio (LMR) by ratio of lymphocyte count to monocyte count; the ratio of platelet count to lymphocyte count provided the platelet lymphocyte ratio (PLR); monocyte leucocyte ratio (MWR) was obtained by ratio of monocyte value to leukocyte value.

Postoperative pathology specimens of the patients were examined. The patients were categorized according to the AFIP [9, 10] risk classification in the pathology specimens. This criterion also considers the anatomical region of the tumor. According to these criteria, in gastric GIST  $\leq 10$  cm and 5 mitoses per 50 HPF have a low risk for metastasis, however 50 HPF  $> 5$  mitosis and  $> 5$  cm diameter have a high risk for metastasis in gastric GIST. On the other hand, regardless of mitotic rate, all intestinal GIST greater than 5 cm are at least moderately at risk for metastases, and the risk of metastasis is known to be high in all  $> 5$  mitoses per 50 HPF. Intestinal GIST  $\leq 5$  mitosis per 50 HPF and  $\leq 5$  cm metastasis risk is low. Immunohistochemical features in the pathology report were removed and their microscopic and macroscopic features were examined. Thirty patients were divided into two groups

according to the AFIP risk scoring system: the group 1 included very low and low risk patients and the group 2 included moderate and high risk patients. Inflammatory indicators LMR, PLR and MWR were compared between the groups. Since the number of cases is limited, all patients were included in the study. There were no excluded cases.

### Statistical analyses

Kolmogorov-Smirnov test was used to check whether the variables were normally distributed or not. While t-test was applied for variables showing normal distribution; Mann-Whitney U tests were used for the analysis of variables not showing normal distribution. The receiver-operating characteristic (ROC) curve was used to identify the optimal cut-off values of statistically significant variables that identified the low-risk patient group. All the analyses were performed with the Statistical Package for Social Sciences 25.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and the results with a level of  $p < 0.05$  were considered to be significant.

### Results

The demographic information and clinicopathological characteristics of 30 patients with GIST are shown in Table 1. The age of the study population was 65.63 (29-86) years. The age of the group 1 was 65.21 (41-85) years and the age of the group 2 was 66 (29-86) years ( $p = 0.75$ ). According to gender, 13 of 30 patients were female and 17 were male. It was seen that 8 of the patients in the group 1 were male and 5 of them were female and 12 of the patients in the group 2 were male and 5 of them were female. There was no significant difference between groups according to the gender of the subjects ( $p = 0.078$ ).

**Table 1.** The demographic information and clinicopathological characteristics of 30 patients with GIST.

Variable	n (%)
Median age (range, yr)	66 (29-86)
Gender	
Female	13 (43)
Male	17 (57)
According to AFIP classification	
Very Low Risk	5 (17)
Low Risk	8 (27)
Moderate	7 (23)
High Risk	9 (30)
Insufficient information	1 (3)
Tumor size (cm)	
$\leq 2$	3 (10)
$>2$ and $\leq 5$	7 (23)
$>5$ and $\leq 10$	11 (37)
$>10$	9 (30)
Tumor location	
Stomach	18 (60)
Jejunum/ileum colon and rectum	12 (40)
Mitosis	
$\leq 5/50$ HPFs	22 (73)
$>5/50$ HPFs	8 (27)
Cellular Type	
Fusiform Cell	18 (60)
Mixed	10 (33)
Epithelioid	2 (7)
Necrosis	
Yes	12 (40)
No	18 (60)
Ulcer	
Yes	12 (40)
No	18 (60)
Bleeding	
Yes	11 (37)
No	19 (63)



When the localization of GIST was examined, it was found that 18 cases were located in the stomach, 12 cases were located in the jejunum / ileum, colon and rectum. One of the patients in Group 1 had GIST located in the small intestine, 12 of them were in the stomach, 11 of the patients in group 2 were located outside the stomach, and 6 of them were located in the stomach.

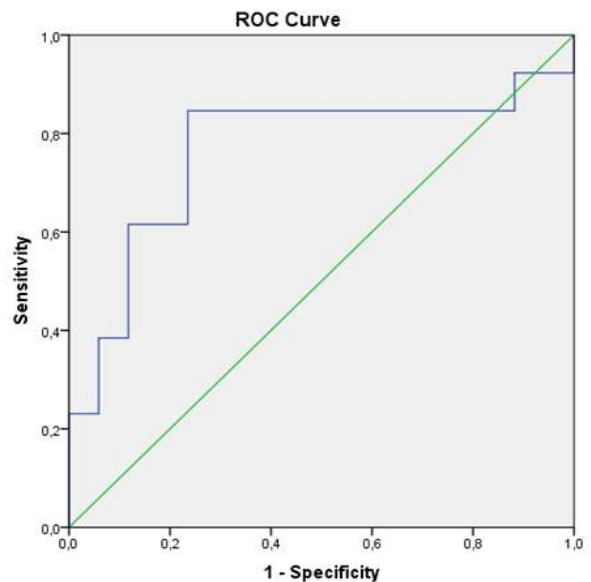
LMR value was significantly higher in group 1 patients ( $5.25 \pm 2.55$ ) than group 2 patients ( $2.92 \pm 1.76$ ) in the preoperative hemogram parameters ( $p = 0.006$ ). PLR of Group 1 (139.68) (77.15-370) ( $p=0.016$ ) was significantly lower than the PLR of Group 2 (185.04) (104.3-430.6) (Table 3). In addition, MWR of group 1 (0.05) (0.03-0.16) was significantly lower than the MWR of group 2 (0.08) (0.04-0.17) ( $p = 0.014$ ).

**Table 2.** Comparisons of preoperative blood test parameters in the groups.

Variables	Group 1 (n=13)	Group 2 (n=17)	<i>p</i>
Neutrophil	4.34(2.29-9.08)	4.85(2.49 -0.74)	0.676
LMR	5.25(±2.55)	2.92 (±1.76)	0.006
MWR	(0.05) (0.03-0.16)	(0.08) (0.04-0.17)	0.014
NLR	4.23 (1.25 - 28.3)	3.84 (1.30 – 8.84)	0.098
PLR	139.6 (77 - 370)	185.04 (104 - 430)	0.016

LMR: lymphocyte monocyte ratio. MWR: monocyte leucocyte ratio. NLR: neutrophil to lymphocyte ratio. PLR: platelet lymphocyte ratio.

ROC analysis was performed to determine the cut-off values of significant LMR values. In patients with GIST, the LMR value above 1.609 level (Figure 1) can be predicting with 84% sensitive and 77% specifically the low or very low risk patients. Area value under the curve was found to be AUC: 0.765.



**Figure 1.** ROC analysis of lymphocyte monocyte ratio (LMR) value.

### Discussion

In this study, LMR was found to be significantly higher in group 1(low and very low-risk GIST) patients compared to group 2 (moderate and high-risk GIST) patients. In addition, LMR value above 1.609 had 84% sensitivity and 77% specificity for low and very low-risk GIST patients. Studies in GIST have focused on determining recurrence or prognosis. Discussions have suggested scoring systems or nomograms to better predict the risk and prognosis of recurrence. These scoring systems or nomograms include mitotic activity, tumor size, and tumor site [10-12] , which are independent prognostic factors. We also used the AFIP scoring system in our study.

Gastric GIST have a lower risk of relapse than non-gastric GIST cases [3, 11]. In our study, tumor site was significantly different between groups 1 and 2. The rate of extra-gastric GIST was higher in the patients with moderate and high risk group ( $p = 0.002$ ).

The use of these criteria aims to identify patients who may benefit from adjuvant

systemic therapy to reduce recurrence. In our study, the AFIP scoring which we evaluated also provides guidance in predicting recurrence after surgery in patients. Patients with low-risk scoring systems usually have positive outcomes and do not require adjuvant therapy [3].

Recent studies aim to predict the prognosis of GIST with inflammatory indicators in blood parameters. Kargin and colleagues [13] evaluated the relationship between elevated blood neutrophil-lymphocyte ratio and prognosis in GIST patients. The authors found that this rate increased significantly in high-risk patients and was associated with short survival [13]. In our study, we found that high LMR values predict low-risk GIST patients.

Studies have shown that systemic inflammatory response and platelets, especially NLR ratio, dNLR (derived Neutrophil Lymphocyte) ratio, LMR ratio and PLR ratio, can predict important clinical outcomes in a wide range of cancers. PLR is also predictive of poor diabetic control in patients with type 1 diabetes mellitus, characterized by low inflammatory burden [14]. Chronic inflammation occurs locally in solid cancers and contributes to tumor growth and progression [15]. LMR, esophageal squamous cell carcinoma [16] and stomach [17], colorectal [18] cancers such as cancers in patients with advanced T stage prognostic importance has been made in studies. In addition, LMR was found to correlate primarily with local cancer progression rather than metastasis and was associated with T stage, similar to other inflammatory markers, but not always with N stage [6]. In the study of LMR in soft tissue sarcomas undergoing curative surgery, it was shown to be an independent prognostic factor in patients [6, 19]. In our study, we found that high LMR values indicate low and very low risk patients in the AFIP scoring system.

In the light of the above statements, the use of adjuvant therapy in low-risk and very low-risk GIST patients was found to be unnecessary, but adjuvant therapy had a place in moderate and high-risk GIST patients. These risk groups can be determined by scoring systems. In our study, we found that the inflammatory indicators LMR, PLR and MWR obtained from preoperative blood values were compatible with AFIP. LMR, PLR and MWR values were statistically significant in predicting low and high risk patients according to AFIP interrogation system. When we evaluate ROC analysis, LMR, PLR and MWR can predict the low and very low risk patients in AFIP scoring system and we just found that the curve of LMR is significant.

The limitation of our study was the limited number of patients. Another limiting factor was the retrospective study. The study should be supported with larger patient series. However, we think that the number of our studies for a rare disease such as GIST will not be underestimated.

### **Conclusions**

As a result of our study, we think that LMR is effective in identifying low and very low risk patients compared to AFIP. From this point of view, we suggest that LMR can identify high and medium risk patients by excluding low and very low risk patients and may be an independent risk factor in GIST scoring systems. We think that low and very low risk patients who do not require adjuvant therapy according to AFIP scoring can be predicted by determining LMR values in the preoperative period.

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**Conflict of Interest:** *The authors declare that they have no conflict of interest.*

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## Effect of single or multiple injection of platelet-rich plasma in comparison with hyaluronic acid on knee osteoarthritis

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### ABSTRACT

**Aim:** To compare the effect of administration of 2 different doses of platelet rich plasma (PRP) and a single dose of hyaluronic acid (HA) preparation on pain and daily life activities of knee osteoarthritis (KOA) patients.

**Method:** In this nonrandomized comparative study, three groups of patients who received either a single dose of intraarticular (IA) PRP (PRP1 group), three doses of IA PRP (PRP3 group), or single dose IA HA (HA group) were included. Assessments were before treatment, and in the 3rd week and 6th week after treatment (after the final injection). The pain-visual analog scale (VAS), Euro-Qol (EQ)-5D-3L, EQVAS, and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) were used.

**Results:** In the 3<sup>rd</sup> week, there were statistically significant differences between the PRP1-HA groups in all parameters except EQ5; between PRP3-HA groups in all parameters except EQ5 and WOMAC stiffness; and between PRP3-PRP1 groups in all parameters except EQVAS, WOMAC pain and WOMAC stiffness. In the 6<sup>th</sup> week, there were statistically significant differences between the PRP1-HA groups in all parameters except WOMAC stiffness; between PRP3-HA groups in all parameters; and between PRP3-PRP1 groups in all parameters except WOMAC pain.

**Conclusion:** Intraarticular PRP injections (single or three doses) were found to be more beneficial in the short term in terms of pain and functional improvement than HA injection and administration of three consecutive doses of PRP may be more effective compared to single-dose PRP administration in KOA patients.

**Keywords:** Knee osteoarthritis, platelet rich plasma, hyaluronic acid, intraarticular injection.

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## Introduction

Osteoarthritis (OA) is the most commonly observed rheumatologic disease in the world resulting from primary progressive cartilage destruction [1]. Variations occurring as a result of OA are the main reason for situations leading to disability and are mostly observed in the knee joints [1-3]. Knee osteoarthritis (KOA) is a progressive joint disease frequently involving intra and periarticular structures characterized by joint cartilage lesions, synovitis, subchondral sclerosis and osteophytes. As a result of these, problems like pain, sensitivity, joint stiffness, swelling in the joint, movement limitation, joint deformity, muscle strength loss, reduced functional capacity and disrupted quality of life may be observed [1-3].

The targets of KOA treatment are to reduce pain, resolve joint stiffness, preserve and improve joint movement, preserve and increase muscle power, prevent trauma or protect against movements that may cause trauma and increase quality of life. Frequently used treatment methods for symptomatic KOA patients before surgery include systemic-effect anti-inflammatory medications, physiotherapy, topical anti-inflammatory gels and intraarticular injections. In spite of medical advances, there is no proven medication or surgical intervention to prevent or delay the development of KOA [3-6].

Intraarticular and periarticular injections have begun to be chosen for KOA treatment in recent years with the aim of improving symptoms and regulating daily life activities. Many studies have reported that hyaluronic acid (HA) has visco-induction properties and may increase the intraarticular viscosity and positively contribute to pain and mobilization. As a result, intraarticular HA injection is commonly used for KOA treatment [7]. Platelet rich plasma (PRP) is obtained by centrifuging full blood and

is the plasma component containing higher concentrations of platelets than full blood [8]. As it contains many growth factors, the use of PRP injections for treatment of a variety of musculo-skeletal system diseases has come to the agenda. Growth factors, considered to affect the healing process, are locally injected into the lesion site with increasing effect on tendon and cartilage tissue regeneration and are stated to have potential use for treatment [9]. The minimal invasive treatment choice of intraarticular PRP injection is commonly used for treatment of clinically associated diseases like KOA. Some publications have proposed that PRP is a more reliable and effective treatment compared to other intraarticular joint injections [10, 11]. Additionally, though intraarticular HA and PRP administration are shown to resolve pain and improve joint functions in patients, there are contradictory publications about the efficacy for KOA patients [12].

PRP and HA injections have increasing areas and frequency of use with every day and are chosen for musculoskeletal system pathologies with different indications. In spite of this frequent use, there is no treatment algorithm prepared based on evidence related to definite indications and administration frequency. Additionally, there are many different brands on the market, and PRP kits with different features and contents and HA preparations which causes further confusion. In our study we compared the effect of administration of 2 different doses of PRP and a single dose of HA preparation on pain and daily life activities of KOA patients.

## Materials and Methods

### Study design

This nonrandomized comparative study was carried out in the Bolu İzzet Baysal Physical

Medicine and Rehabilitation Training and Research Hospital, after Ethical Committee approval (Usak University Medical School Ethics Committee, decision number 31-5-13, dated 2018/04/25). The study protocol abided by the principles of the Helsinki Declaration. Participants in the research first read and then signed the consent form.

### **Participants**

The study included patients attending the Physiotherapy and Rehabilitation Clinic from January 2019-January 2020 with diagnosis of KOA who received knee intraarticular PRP or HA treatment and agreed to complete the survey forms.

Inclusion criteria for the study were age over 30 years, gonarthrosis diagnosis according to American College of Rheumatology (ACR) criteria [3], and cases identified as stage 1-2-3 according to radiological Kellgren-Lawrence classification [12].

Exclusion criteria for the study were presence of inflammatory rheumatologic disease, coagulation disorder, and immunosuppressive disease, diseases causing disruption to hemogram parameters, serious cardiovascular disease, previous operation in the knee region, varus and valgus deformity of the knee region, malignancy, infection, anticoagulant medication use, and use of anti-inflammatory medication in the last 1 week.

A total of 278 patients were assessed for the study. The study included 210 patients abiding by the study criteria and providing consent with the patient information form (Figure 1). The study grouped patients according to the treatment they received; 70 patients with a single dose of intraarticular (IA) PRP (PRP1 group), 70 patients with three doses of IA PRP (PRP3 group) and 70 patients with single dose IA HA (HA group). It was not possible to blind the patients due to the design of the study and

nature of the treatment. The outcome assessment process was blinded. Patient assessment and statistical analysis of outcomes were performed by a clinician and biostatistics expert blind to the treatments and groups of patients.

### **Interventions**

In our study, all injections performed by a single clinician in the injection clinic under sterile conditions. IA injection used a single-use 10 mL 21 G green-tip injector with the lateral approach in the suprapatellar region. In our clinic, PRP was administered either as single dose or three doses with one-week interval; this approach was previously investigated in Görmeli et al.'s study [11].

The PRP1 group had one single IA PRP dose administered. The PRP amount was 3 mL. Before injection, and in the 3<sup>rd</sup> and 6<sup>th</sup> weeks after injection patients were assessed in terms of pain and functional status.

The PRP3 group had three doses of IA PRP administered at one-week intervals. The amount of PRP administered in each session was 3 mL. Before injection, and in the 3<sup>rd</sup> and 6<sup>th</sup> weeks after injection patients were assessed in terms of pain and functional status.

The HA group had a single dose of IA HA injection administered. Before injection, and in the 3<sup>rd</sup> and 6<sup>th</sup> weeks after injection patients were assessed in terms of pain and functional status.

All patients with PRP administration used a Dr PRP<sup>®</sup> Kit with FDA approval and CE certification offered to the market by Cureacell Ltd. Co. The kit is offered to the market after gamma ray sterilization according to ISO 13485 standards. For preparation of the Dr.PRP kit<sup>®</sup>, 3–4 ml of PRP with a concentration of 8–10 times the average normal value and 2 cc of anti-coagulant were placed in a 20 cc syringe, then 18 cc of blood from patient was drawn.

The drawn blood was injected into the Dr. PRP<sup>®</sup> kit through the upper injection port until the blood level reaches the 20-cc scale marked on the kit. After the first centrifugation at 3000 rpm for 3-4 mins, the plasma layer and the red blood cell (RBC) layer were separated and then the separation position of the plasma and the RBC layer were identified and the height of the separated boundary to the indicated point was adjusted by pushing up or pulling down the adjusting knob located at the lower part of the Kit. In order to block the plasma and the RBC layer completely, the adjusting knob and the valve were fastened (clockwise). Finally, the adjusting knob was fastened again. The fastened PRP Kit was put into the centrifuge with counterbalance for the second centrifugation to enrich the concentrated platelets at 3250 rpm for 4-6 mins. The PRP Kit was placed in upright position and the upper silicone lid on the Kit was opened. The PPP (platelet poor plasma) layer was slowly removed from the upper part using a 10-cc syringe with a needle, leaving 3 cc in the lower part (PRP). The PRP preparation procedure was performed by a trained nurse in our clinic.

Patients with IA HA administered used the product with CE, ED and REP certification sold as ArtıAid<sup>®</sup> Plus Intra-articular Injection commercial brand by Maxıgen Biotech Inc. High-purity HA has more than 1,500 kDa molecular weight with 45 mg HA (1.5%) included in 3 mL sodium hyaluronate solution prepared with buffered physiological saline in a single use sterile injector.

During the treatment, patients were told they could use local ice compression and paracetamol (max 2 g/day) if required. Additionally, patients were given a home exercise program and recommended to return to normal daily activities 3 days after injection if tolerated.

### ***Instruments***

Assessments were before treatment, and in the 3<sup>rd</sup> week and 6<sup>th</sup> week after treatment (after the final injection). Assessments used the pain-visual analog scale (VAS), Euro-Qol (EQ)-5D-3L, EQ VAS, and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC).

VAS is a commonly used method to determine the degree of pain. It comprises a line with 100 mm length drawn on a horizontal or vertical axis. The distance from the lowest VAS value to the point indicated by the patient is measured in mm (0-100) and a numerical value is determined for the severity of pain felt by the patient [13].

The 3-level version of EQ-5D (EQ-5D-3L) was developed by the European Quality of Life (Euroqol) Group in 1990. The EQ-5D-3L comprises 2 pages of the EQ-5D descriptive system and EQ visual analog scale (EQ-VAS). The EQ-5D is defined in terms of 5 subdimensions (mobility, self-care, general activities, pain/discomfort and anxiety/depression) within a three-level structure of “no problem, moderate degree problems and advanced degree problems”. On scoring a value of 1 shows perfect health, while health status worsens as values reduce. The EQ-VAS comprises a 100 mm line to assist in scoring the health status of a person with the best health status imaginable shown at 100 and the worst health status shown at 0 [14].

The WOMAC is a health status metric commonly used for knee and hip OA patients. It comprises three sections of pain, stiffness and physical function. It includes a total of 24 items. Points for items are given according to a Likert scale. Points from 0 to 4 are given on the Likert scale determining pain and degree of difficulty. Turkish validity and reliability studies have been performed [15].



Socio-demographic (or other) variables such as age, gender and symptom duration (months) were recorded in all patients.

### Statistical methods

The baseline characteristics were compared among groups by using the Kruskal-Wallis test or the Mann-Whitney U test for continuous variables and Pearson's chi-square test for categorical variables. Outcomes were analyzed with generalized linear mixed models with gamma regression. The models included group, time, some baseline characteristics (i.e. age, sex, OA grade), baseline value of outcome and group X time interaction as fixed effects. Follow-up and difference values are presented as generalized linear mixed models estimated mean (95% confidence interval). The sequential Bonferroni correction was used in the models. All statistical analyses were performed using SPSS. The level of statistical significance was set at 0.05.

### Results

Of the total of 210 patients (70 x 3 groups) included within the scope of the study, the study was completed with 66 people in the PRP1 group, 65 people in the PRP3 group and 68 people in the HA group. In the PRP1 group, 3 people did not continue to attend check-ups and 5 people used NSAIDs; in the PRP3 group 6 people used NSAIDs, 3 people ended participation after one or two injections, 1 person developed history of trauma during follow-up and 2 people had arthroscopic surgery; and in the HA group 3 people did not continue to attend check-ups so the study was completed with a total of 176 patients. The flow diagram for the study is presented in Figure 1. The basic descriptive characteristics of patients are summarized in Table 1.

The mean difference in pain VAS scores between the groups was identified to be

statistically significant in the 3<sup>rd</sup> week. Estimated mean differences were -4.01 (95% CI, -6.86 to -1.16;  $p=0.006$ ) between PRP1 and HA groups, -8.42 (95% CI, -11.87 to -4.97;  $p<0.001$ ) between PRP3 and HA groups and -4.41 (95% CI, -7.65 to -1.18;  $p=0.005$ ) between PRP3 and PRP1 groups. The mean difference between pain VAS scores between the groups was identified to be statistically significant in the 6<sup>th</sup> week. The estimated mean differences were -6.31; 95% CI, -8.66 to -3.97;  $p<0.001$ , between PRP1 and HA groups, -9.86; 95% CI, -12.34 to -7.39;  $p<0.001$  between PRP3 and HA groups and -3.55; 95% CI, -5.27 to -1.83;  $p<0.001$ ) between PRP3 and PRP1 groups (Table 2, Figure 2).

Mean differences between the EQ5 scores in the groups was only identified to be statistically significant between the PRP3 and PRP1 groups in the 3<sup>rd</sup> week. The estimated mean differences were -0.21; 95% CI, -0.06 to 0.019;  $p=0.303$  between PRP1 and HA groups, 0.03; 95% CI, -0.10 to 0.078;  $p=0.132$  between PRP3 and HA groups and 0.06; 95% CI, 0.01 to 0.10;  $p=0.016$  for PRP3 and PRP1 groups. In the 6<sup>th</sup> week, the estimated mean differences were 0.14; 95% CI, 0.07 to 0.21;  $p<0.001$  between PRP1 and HA groups, 0.24; 95% CI, 0.16 to 0.31;  $p<0.001$  between PRP3 and HA groups and 0.10; 95% CI, 0.02 to 0.18;  $p=0.006$  for PRP3 and PRP1 groups (Table 2, Figure 2).

The mean differences between the EQ VAS scores in the groups was identified to be statistically significant between the PRP1-HA and PRP3-HA groups in the 3<sup>rd</sup> week. The estimated mean differences were 5.67; 95% CI, 2.02 to 9.32;  $p=0.001$  between PRP1 and HA groups, 5.86; 95% CI, 2.11 to 9.60;  $p=0.001$  between PRP3 and HA groups and 0.19; 95% CI, -3.15 to 3.52;  $p=0.913$  for PRP3 and PRP1 groups. Statistical significance was identified for the mean differences between groups for EQ

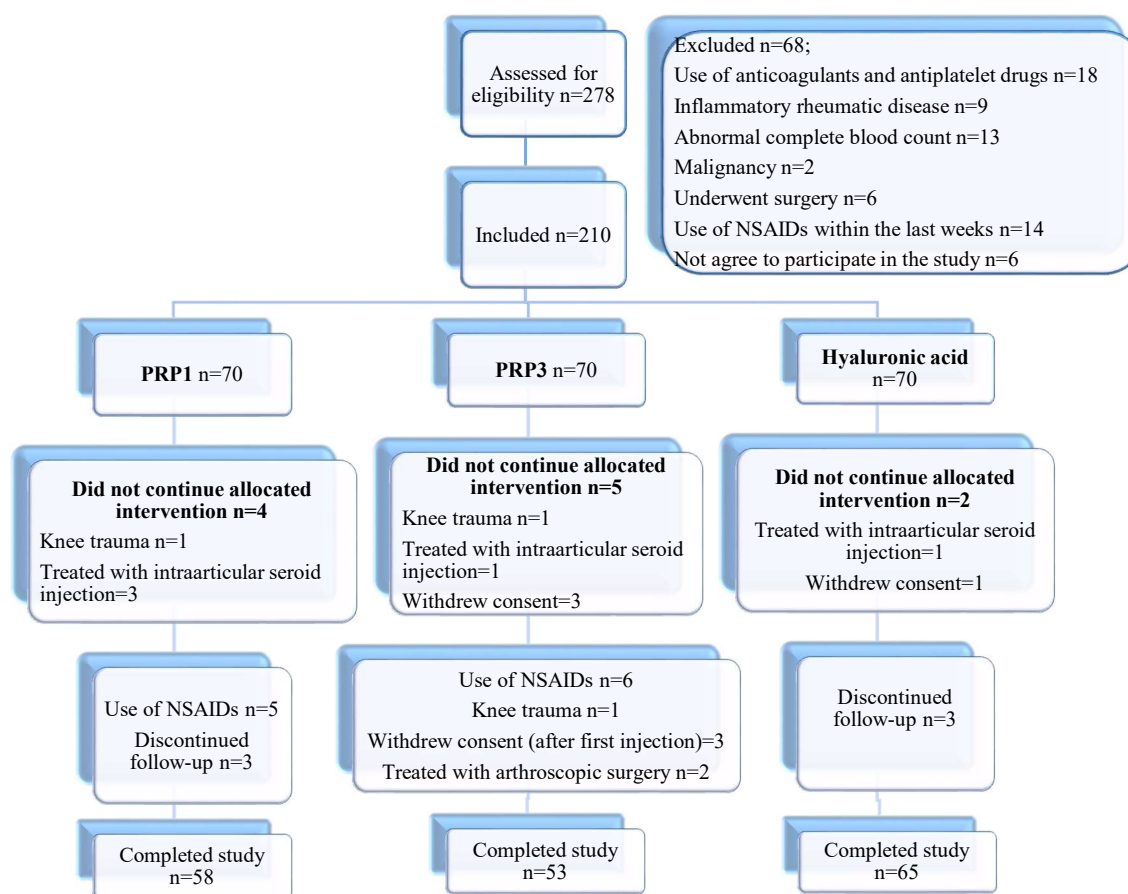


Figure 1. Flow diagram of the study population

Table 1. Baseline characteristics of patients†.

Parameters	PRP1	PRP3	Hyaluronic acid	P value‡	P value§		
					PRP1-HA	PRP3-HA	PRP3-PRP1
Age, years	46.52 ± 11.22	43.49 ± 12.06	49.18 ± 12.64	0.042	0.185	0.016	0.159
Female sex	32 (55.2%)	29 (54.7%)	34 (52.3%)	0.943	NA	NA	NA
Kellgren-Lawrence							
Grade 1	23 (39.7%)	19 (35.8%)	18 (27.7%)	0.158	NA	NA	NA
Grade 2	28 (48.3%)	22 (41.5%)	27 (41.5%)				
Grade 3	7 (12.1%)	12 (22.6%)	20 (30.8%)				
Body mass index (kg/m <sup>2</sup> )	26.95 ± 3.64	26.68 ± 3.42	27.54 ± 3.32	0.230	NA	NA	NA
Duration (Years)	4.38 ± 1.14	4.62 ± 1.37	4.86 ± 1.78	0.341	NA	NA	NA
VAS Pain	72.50 ± 9.56	80.66 ± 12.86	75.23 ± 10.13	0.001	0.216	0.007	<0.001
EQ5	0.16 ± 0.22	0.09 ± 0.22	0.09 ± 0.19	0.068	NA	NA	NA
EQ VAS	27.50 ± 9.56	18.96 ± 12.38	24.77 ± 10.13	<0.001	0.063	0.004	0.037
Womac Pain	11.29 ± 2.29	13.42 ± 3.10	12.45 ± 2.23	<0.001	0.002	0.023	<0.001
Womac Stiffness	3.86 ± 1.07	4.91 ± 1.26	4.14 ± 1.00	<0.001	0.105	0.001	<0.001
Womac Function	46.86 ± 7.43	52.70 ± 9.34	49.14 ± 7.47	<0.001	0.031	0.010	<0.001
Womac Total	61.98 ± 10.33	71.15 ± 13.34	65.69 ± 9.60	<0.001	0.016	0.004	<0.001

† The data are expressed as mean ± standard deviation or number (%). PRP: Platelet-Rich Plasma, VAS: Visual Analog Scale, EQ: European Quality of life, WOMAC: Western Ontario and Mc Master Universities Osteoarthritis index, NA: Not applicable.

‡ The Kruskal-Wallis test was used for continuous variables; and Pearson's chi-square test was used for categorical variables between three groups (PRP1, PRP3, HA).

§ The Mann-Whitney U test was used for continuous variables between two groups.

**Table 2.** Outcomes for PRP1, PRP3 and HA groups at 3 week and 6 week †

	Treatment difference						Pvalue‡		
	Platelet-Rich Plasma-1 (PRP1)	Platelet-Rich Plasma-3 (PRP3)	Hyaluronic acid (HA)	PRP1-HA	PRP3-HA	PRP3-PRP1	PRP1-HA	PRP3-HA	PRP3-PRP1
<b>Pain VAS</b>									
Baseline	72.50 ± 9.56	80.66 ± 12.86	75.23 ± 10.13						
3 week	35.99 (33.98 to 38.12)	31.57 (29.74 to 33.52)	40.00 (38.00 to 42.10)	-4.01 (-6.86 to -1.16)	-8.42 (-11.87 to -4.97)	-4.41 (-7.65 to -1.18)	0.006	<0.001	0.005
6 week	11.83 (10.65 to 13.15)	8.28 (7.19 to 9.54)	18.14 (16.58 to 19.86)	-6.31 (-8.66 to -3.97)	-9.86 (-12.34 to -7.39)	-3.55 (-5.27 to -1.83)	<0.001	<0.001	<0.001
<b>EQ5</b>									
Baseline	0.16 ± 0.22	0.09 ± 0.22	0.09 ± 0.19						
3 week	0.44 (0.41 to 0.47)	0.49 (0.46 to 0.53)	0.46 (0.43-0.49)	-0.21 (-0.06 to 0.019)	0.03 (-0.10 to 0.078)	0.06 (0.01 to 0.10)	0.303	0.132	0.016
6 week	0.88 (0.83 to 0.94)	0.98 (0.92-1.04)	0.74 (0.70-0.79)	0.14 (0.07 to 0.21)	0.24 (0.16 to 0.31)	0.10 (0.02 to 0.18)	<0.001	<0.001	0.016
<b>EQ VAS</b>									
Baseline	27.50 ± 9.56	18.96 ± 12.38	24.77 ± 10.13						
3 week	65.05 (62.79 to 67.40)	65.24 (62.88 to 67.68)	59.38 (57.46 to 61.36)	5.67 (2.02 to 9.32)	5.86 (2.11 to 9.60)	0.19 (-3.15 to 3.52)	0.001	0.001	0.913
6 week	89.24 (87.39 to 91.12)	95.92 (93.89 to 97.99)	81.03 (79.54 to 82.55)	8.21 (5.54 to 10.87)	14.89 (11.74 to 18.05)	6.69 (3.89 to 9.48)	<0.001	<0.001	<0.001
<b>Womac Pain</b>									
Baseline	11.29 ± 2.29	13.42 ± 3.10	12.45 ± 2.23						
3 week	6.23 (5.82 to 6.67)	6.12 (5.72 to 6.56)	7.60 (7.16 to 8.06)	-1.37 (-2.06 to -0.68)	-1.48 (-2.24 to -0.71)	-0.11 (-0.71 to 0.50)	<0.001	<0.001	0.729
6 week	1.99 (1.75 to 2.27)	1.66 (1.46 to 1.89)	3.73 (3.32 to 4.19)	-1.74 (-2.31 to -1.17)	-2.07 (-2.66 to -1.48)	-0.33 (-0.671 to 0.003)	<0.001	<0.001	0.052
<b>Womac Stiffness</b>									
Baseline	3.86 ± 1.07	4.91 ± 1.26	4.14 ± 1.00						
3 week	2.00 (1.83 to 2.17)	2.08 (1.91 to 2.27)	2.34 (2.17 to 2.52)	-0.34 (-0.63 to -0.06)	-0.25 (-0.55 to 0.04)	0.09 (-0.16 to 0.34)	0.013	0.104	0.492
6 week	1.08 (0.94 to 1.23)	0.81 (0.68 to 0.96)	1.23 (1.12 to 1.36)	-0.16 (-0.34 to 0.03)	-0.43 (-0.66 to -0.20)	-0.27 (-0.51 to -0.04)	0.099	<0.001	0.019
<b>Womac Function</b>									
Baseline	46.86 ± 7.43	52.70 ± 9.34	49.14 ± 7.47						
3 week	27.47 (26.10 to 28.91)	25.03 (23.77 to 26.36)	31.57 (30.17 to 33.03)	-4.10 (-6.32 to -1.87)	-6.54 (-8.92 to -4.15)	-2.44 (-4.36 to -0.52)	<0.001	<0.001	0.013
6 week	8.94 (7.99 to 9.99)	6.88 (6.13 to 7.73)	15.82 (14.26 to 17.55)	-6.88 (-9.07 to -4.69)	-8.94 (-11.18 to -6.70)	-2.05 (-3.33 to -0.78)	<0.001	<0.001	0.002
<b>Womac Total</b>									
Baseline	61.98 ± 10.33	71.15 ± 13.34	65.69 ± 9.60						
3 week	35.77 (34.02 to 37.62)	32.79 (31.15 to 34.52)	41.52 (39.71 to 43.41)	-5.74 (-8.61 to -2.88)	-8.73 (-11.82 to -5.63)	-2.98 (-5.47 to -0.49)	<0.001	<0.001	0.019
6 week	11.28 (10.09 to 12.62)	8.59 (7.65 to 9.65)	20.32 (18.31 to 22.56)	-9.04 (-11.85 to -6.23)	-11.73 (-14.60 to -8.86)	-2.69 (-4.31 to -1.08)	<0.001	<0.001	0.001

† Baseline data are mean ± standard deviation. Follow-up and difference values are generalized linear mixed models estimated mean (95%CI). The models included group, time, some baseline characteristics (i.e. age, sex, OA grade), baseline value of outcome and group X time interaction as fixed effects. †/AS Visual Analog Scale, †/OMAC Western Ontario and Mc Master Universities Osteoarthritis index. ‡ Generalized linear mixed models adjusted (sequential Bonferroni) P value.

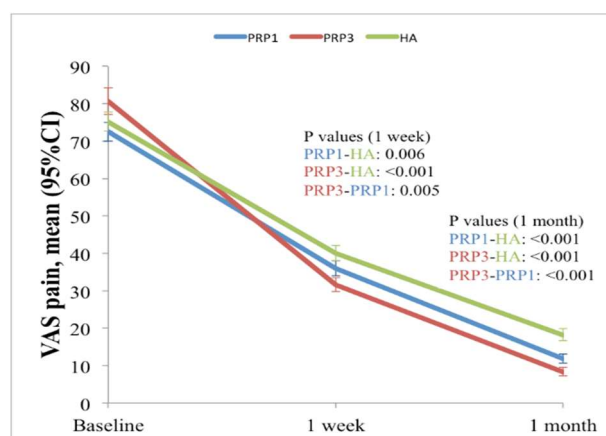
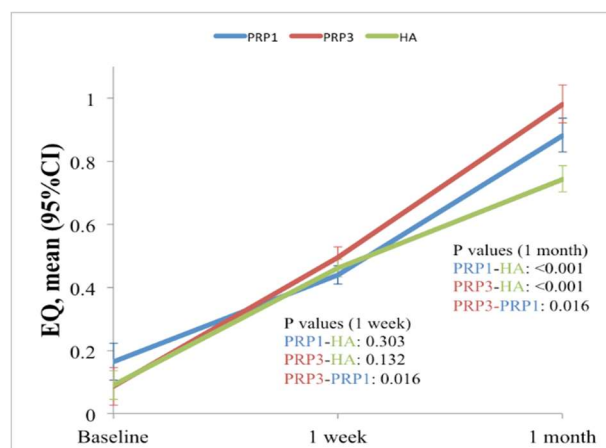
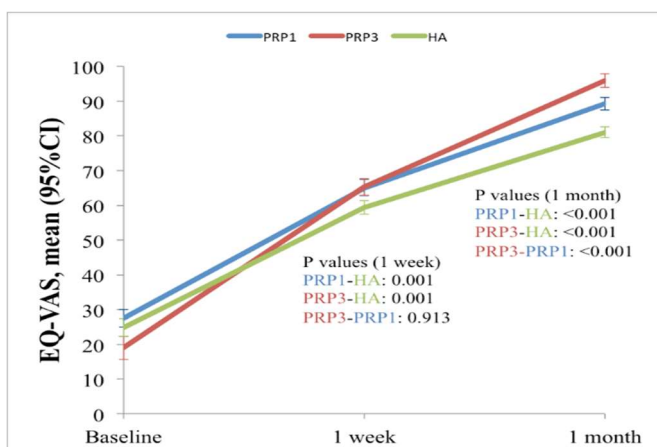
VAS scores in the 6<sup>th</sup> week. The estimated mean differences were 8.21; 95% CI, 5.54 to 10.87;  $p < 0.001$  between PRP1 and HA groups, 14.89; 95% CI, 11.74 to 18.05;  $p < 0.001$  between PRP3 and HA groups and 6.69; 95% CI, 3.89 to 9.48;  $p < 0.001$  for PRP3 and PRP1 groups (Table 2, Figure 2).

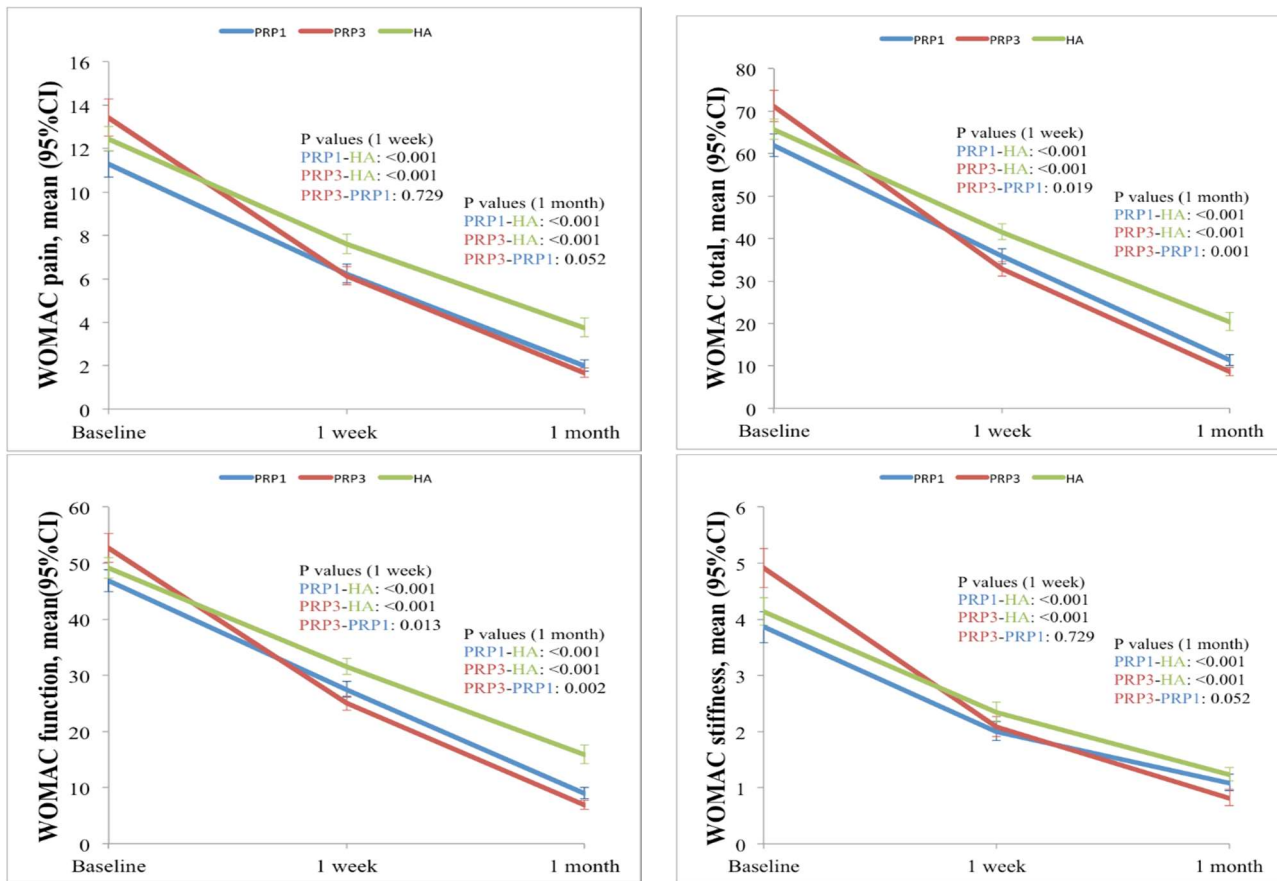
There were statistically significant differences between the PRP1-HA and PRP3-HA groups in the 3<sup>rd</sup> week for the WOMAC-pain scores in the groups. The estimated mean differences were 1.37; 95% CI, -2.06 to -0.68;  $p < 0.001$  between PRP1 and HA groups, -1.48; 95% CI, -2.24 to -0.71;  $p < 0.001$  between PRP3 and HA groups and -0.11; 95% CI, -0.71 to 0.50;  $p = 0.729$  for PRP3 and PRP1 groups. Differences between the mean WOMAC-pain scores between the groups in the 6<sup>th</sup> week were identified to be statistically significant between the PRP1-HA and PRP3-HA groups. The estimated mean

differences were -1.74; 95% CI, -2.31 to -1.17;  $p < 0.001$  between PRP1 and HA groups, -2.07; 95% CI, -2.66 to -1.48;  $p < 0.001$  between PRP3 and HA groups and -0.33; 95% CI, -0.671 to 0.003;  $p = 0.052$  for PRP3 and PRP1 groups (Table 2, Figure 2).

The mean differences between WOMAC-stiffness scores in the groups in the 3<sup>rd</sup> week were identified to be statistically significant for the PRP1-HA groups. The estimated mean differences were 0.34; 95% CI, -0.63 to -0.06;  $p = 0.013$  between PRP1 and HA groups, -0.25; 95% CI, -0.55 to 0.04;  $p = 0.104$  between PRP3 and HA groups and 0.09; 95% CI, -0.16 to 0.34;  $p = 0.462$  for PRP3 and PRP1 groups. In the 6<sup>th</sup> week, mean differences between the WOMAC-stiffness scores in the groups were identified to be statistically significant between the PRP3-PRP1 and PRP3-HA groups. The estimated mean differences were -0.16; 95% CI, -0.34 t

**Figure 2.** Baseline values are mean (95%CI). Follow-up values are generalized mixed models estimated mean (95%CI). The models included group, time, some baseline characteristics (i.e. age, sex, OA grade), baseline value of outcome and group X time interaction as fixed effects. VAS: Visual Analog Scale, EQ: European Quality of life, WOMAC: Western Ontario and Mc Master Universities Osteoarthritis index. Generalized linear mixed models adjusted (sequential Bonferroni) *P values* are presented.





0.03;  $p=0.099$  between PRP1 and HA groups, 0.43; 95% CI, -0.66 to -0.20;  $p<0.001$  between PRP3 and HA groups and -0.27; 95% CI, -0.51 to -0.04;  $p=0.019$  for PRP3 and PRP1 groups (Table 2, Figure 2).

There were statistically significant differences between the groups in the 3<sup>rd</sup> week for the WOMAC-function scores. The estimated mean differences were -4.10; 95% CI, -6.32 to -1.87;  $p<0.001$  between PRP1 and HA groups, -6.54; 95% CI, -8.92 to -4.15;  $p<0.001$  between PRP3 and HA groups and -2.44; 95% CI, -4.36 to -0.52;  $p=0.013$  for PRP3 and PRP1 groups. Differences between the mean WOMAC-function scores between the groups in the 6<sup>th</sup> week were identified to be statistically significant. The estimated mean differences were -6.88; 95% CI, -9.07 to -4.69;  $p<0.001$  between PRP1 and HA groups, -8.94; 95% CI, -11.18 to -6.70;  $p<0.001$  between PRP3 and HA groups and -2.05; 95% CI, -3.33 to -0.78;

$p=0.002$  for PRP3 and PRP1 groups (Table 2, Figure 2).

The mean difference between WOMAC-total scores in the groups in the 3<sup>rd</sup> week were identified to be statistically significant. The estimated mean differences were -5.74; 95% CI, -8.61 to -2.88;  $p<0.001$  between PRP1 and HA groups, -8.73 95% CI, -11.82 to -5.63;  $p<0.001$  between PRP3 and HA groups and -2.98; 95% CI, -5.47 to -0.49;  $p=0.019$  for PRP3 and PRP1 groups.

In the 6<sup>th</sup> week, mean differences between the WOMAC-total scores in the groups were identified to be statistically significant. The estimated mean differences were -9.04; 95% CI, -11.85 to -6.23;  $p<0.001$  between PRP1 and HA groups, -11.73; 95% CI, -14.60 to -8.86;  $p<0.001$  between PRP3 and HA groups and -2.69; 95% CI, -4.31 to -1.08;  $p=0.001$  for PRP3 and PRP1 groups (Table 2, Figure 2).

## Discussion

The study found statistically significant differences between the PRP1-HA groups in all parameters except EQ5, between PRP3-HA groups in all parameters except EQ5 and WOMAC stiffness, and between PRP3-PRP1 groups in all parameters except EQVAS, WOMAC pain and WOMAC stiffness in the 3<sup>rd</sup> week; and statistically significant differences between the PRP1-HA groups in all parameters except WOMAC stiffness; between PRP3-HA groups in all parameters; and between PRP3-PRP1 groups in all parameters except WOMAC pain in the 6<sup>th</sup> week.

The targets of treatment for KOA include controlling pain, minimizing physical limitations, increasing quality of life and if possible, stopping progression of pathological processes [3-6]. Treatment should be specifically organized according to each individual based on patient expectations, disease severity, activity level and presence of comorbid diseases [3-6]. The minimal invasive treatments of intraarticular HA and PRP administration are commonly used treatment alternatives. Though many studies have been published about both treatment methods, effects and efficacy are still controversial [8-12].

In KOA treatment, just as with IA PRP injection, the use of autologous growth factors is increasing [16]. PRP is the most convenient agent to obtain when compared with products containing other autologous growth factors. PRP contains factors like platelet-derived insulin-like growth factor, fibroblast growth factor, platelet-derived growth factor, epidermal growth factor and venous endothelial growth factor. These factors obtained from PRP may change the inflammatory process and have been shown to assist in preserving and regenerating tissue structure [17,18]. Due to

these features, PRP is used in many different areas, not just for joint pathologies [19]. PRP contributes to the repair processes in subchondral bone and cartilage in KOA [20]. It reduces the negative effects of knee pain and inflammatory response [21]. Many reviews have reported positive clinical effects of PRP injection. PRP was shown to reduce pain and improve osteoarthritis indices (WOMAC total score, WOMAC subscores and Lequesne score) in KOA patients [22-29].

PRP injection is observed to be effective in early symptomatic OA knees. Outcomes after treatment show a clear reduction in pain in the 12<sup>th</sup> month compared to situation before treatment and continued improvement in knee functions [22]. A study of patients with moderate stage KOA administered a single injection of PRP and two and three doses of PRP at two-week intervals and analyzed results at the end of the 6<sup>th</sup> month. In conclusion, they showed that for improvement in functional status and pain, a minimum of two injections were required [30]. A study of late stage (stage IV) KOA patients with single dose PRP and single dose steroid injection identified that the daily life activities, pain and QoL scores were similar in the two groups in the 6<sup>th</sup> month, with a significant improvement compared to initially [31].

A meta-analysis included many studies researching the clinical effect of PRP and stated that PRP was effective for KOA treatment but there was no clear evidence about dose or frequency. In this study, Vilchez-Cavazos et al. assessed 6-month outcomes and stated that single dose PRP had similar levels of improvement in terms of pain to multiple PRP doses; however, multiple dose PRP groups had more significant improvement in terms of joint functions [32]. Patel et al. compared efficacy at the end of the 6<sup>th</sup> month for 1 and 2 doses of

PRP with single-dose saline injection and showed that PRP injections ensured better improvement compared to saline injections in terms of WOMAC scores; however, there was no difference between the two PRP injections [33]. Görmeli et al. showed that three doses of PRP injection provided significantly better improvement compared with a single injection for early OA (stage I, II, III) patients; however, in advanced OA patients (stage IV) there was no difference between the groups [11]. In our study, early and moderate stage KOA patients (stage I, II, III) had the short-term effects of PRP injection investigated and both PRP groups had independent improvement identified in terms of pain (VAS), quality of life scores (EQ-5D) and daily life activities (WOMAC). The group with 3 consecutive PRP injections were identified to have significant improvement in the 3<sup>rd</sup> and 6<sup>th</sup> weeks compared to the PRP1 group.

Significant problems experienced with PRP administration may be listed as obtaining PRP solution amounts, platelet concentration in contents, use of tubes and kits with different features, homogenization of obtained PRP and user experience [34,35]. In our study, a PRP kit abiding by standardization as determined by the Turkish Ministry of Health and international standards and with safety certification was used. The PRP solutions for administration were prepared by an experienced health staff with clinical training and administered by a single clinician.

Patients with HA injection, assessed in many studies for knee treatments, were not identified to have any difference compared to patients with single-dose PRP injection. Patients with multiple PRP doses were identified to have greater improvement than patients with one of the other two treatments administered [34, 36-40].

In the literature, though studies comparing PRP and HA injections and meta-analyses have generally emphasized that IA PRP administration is more effective compared to HA administration in terms of pain and functional improvement [23, 27, 29, 41-43], a few meta-analyses have reported the opposite view [44, 45]. PRP injection was shown to be more effective in reducing symptoms in mild and moderate (stage I, II, III) KOA patients who do not respond to traditional treatment and in improving function and quality of life compared to HA injection and placebo in many studies in the literature [42, 46-49].

Görmeli et al. in a study of PRP and HA injections showed that there were significant degrees of improvement in early OA (stage I, II, III) patients in terms of pain and function improvement; however, there was no difference between the groups for advanced OA (stage IV) patients [34]. Zhang et al. compared pain, function and quality of life indices after PRP and HA injections and showed that patients in different stages of KOA did not show the same response to PRP or HA treatment [12]. Kon et al. investigated three homogeneous patient groups treated with PRP, low-molecular weight HA and high molecular weight HA and concluded that autologous PRP injections had longer duration of efficacy compared to HA injections and improved joint functions [47]. In our study, early and moderate stage (stage I, II, III) KOA patients had single dose and triple dose of PRP and high-molecular weight HA administered IA. There are standardization problems with PRP kits and the treatment performed with these kits and with HA preparations. Products offered for use may be obtained with different technological methods, have different molecular weights and doses, and have problems like being straight or cross-linked causing different treatment outcomes

and complications to be encountered. In our study, all patients had PRP kit and HA preparations administered with the same brands and features. No infection or allergic reactions were encountered during follow-up. In the 3<sup>rd</sup> and 6<sup>th</sup> weeks after injections, scores indicating pain, quality of life and daily life activities were improved in all groups. This improvement was identified to be at more significant levels in the PRP groups compared to the HA group. When the PRP groups are compared, all scores in the PRP3 group were significantly better than the PRP1 group. In our study, we think the short-term efficacy of PRP injections is due to symptomatic amelioration occurring with physiological variations effective on pain in the intra/periarticular region, rather than positive changes to the pathologic degeneration process in the joint structure or knee OA. However, the improvement after PRP treatment compared to HA treatment, more pronounced after three doses of PRP, leads to consideration that the regeneration process begins in the short term. In order to reveal regenerative changes after PRP administration, it is necessary to perform moderate and long-term follow-up with radiological and histopathological investigations needed to prove these changes. The nonrandomized design, the patient follow-up duration being limited to 6 weeks, and not showing the presence of regeneration after the administered treatments with histopathologic and/or imaging methods may be listed as important limitations of our study. Also, the lack of recording the adherence to home exercise program is another limitation: patients who adhered to home exercise program might have been better improvements than those who did not adhere to it.

### **Conclusions**

Intraarticular PRP injections (single or three doses) were found to be more beneficial in the

short term in terms of pain and functional improvement than HA injection and administration of three consecutive doses of PRP may be more effective compared to single-dose PRP administration in KOA patients

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**Ethical statement:** *All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Research Ethics Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants prior to being included in the study.*

*The study was approved by Usak University Medical School Ethics Committee, decision number 31-5-13, dated 2018/04/25.*

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## Prognostic value of optic nerve sheath thickness in patients with central and peripheral vertigo

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### ABSTRACT

**Aim:** To evaluate the role of the diameter of the optic nerve sheath (ONSD) in the differential diagnosis of the central and peripheral vertigo in patients, who had applied with the complaints of vertigo.

**Method:** Our study had a prospective design and 113 vertigo patients were included in the study. The demographic characteristics, vital signs, symptoms accompanying vertigo and findings of the imaging examinations were evaluated.

**Results:** The median age of our patients was 43 years (IQR: 17) and 44.2 % of them were males. 19.5 % of the patients were diagnosed with central and 80.5 % with peripheral vertigo. In our study, the median ONSD was 4.88 mm (IQR=0.86) in patients with central vertigo and 4.65 mm (IQR=0.20) in patients with peripheral vertigo. The median value of ONSD in patients with central vertigo was significantly higher ( $p=0.030$ ). In our study, the area under the curve was 0.654 (95 % CI=0.498-0.810) and the sensitivity and specificity for the cut-off value of 4.65 mm were 68.2 % and 61.5 % respectively.

**Conclusion:** We determined that ONSD was larger in patients with central vertigo. Further studies with larger subject size are needed on this topic.

**Keywords:** Central vertigo, peripheral vertigo, emergency, optic nerve sheath thickness.

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### Introduction

Vertigo is in the top 10 among the admissions to the emergency units. Vertigo is a dizzying sensation of being in tilting or spinning surroundings. The rate of the lifetime and yearly prevalence of vertigo are 7.4 % and 4.9 % respectively [1].

40-60 % of all vertigo cases are caused by the peripheral vestibular dysfunction, 10-20 % by the central causes, 1-2 % by the medication, and 15 % by the psychiatric disorders. 10 % of the cases are caused by unknown pathological conditions [2,3].

It was determined that one-third of the patients, who were discharged from the emergency units with the diagnosis of peripheral vertigo, had posterior ischemia. The rate of the morbidity and mortality increase in patients with the wrong diagnosis due to the lack of an appropriate treatment [4]. It has been

demonstrated that the brain perfusion is impaired and the intracranial pressure is increased in several cerebrovascular disorders, which may cause vertigo [4].

Hayreh had shown in 1978 that the optic nerve sheath diameter (ONSD) was dilated in patients with increased intracranial pressure (ICP). Following this study, the physicians started to focus on the determination of the ICP with non-invasive methods [5]. In the recent studies, the importance of ONSD in the diagnosis of ICP was evaluated in the disorders (e.g. cranial trauma, stroke, intracranial mass lesion, infection etc.) accompanied by the increased ICP and encouraging results were obtained [6-9]. There are no publications in the literature regarding the ONSD in the differential diagnosis of the central and peripheral vertigo. In our study, our objective was to evaluate the role of ONSD in the differential diagnosis of central and peripheral vertigo in patients, who applied with the complaint of vertigo.

### Materials and Methods

Following the approval of the Ethics Committee of the Medical Faculty at Bolu Abant İzzet Baysal University (Date: 08/02/2018; Decision number: 2018/13), 113 volunteered patients, who had applied to our emergency clinic between 01.02.2018-31.07.2018 and had isolated vertigo, were investigated in a prospective design. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Research Ethics Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all participants prior to being included in the study. We established a standardized form for data collection. The demographic characteristics

(age, gender), vital signs (systolic and diastolic blood pressure and heart rate at admission) and symptoms of the patients, who were enrolled into the study, were recorded. In the patients with central vertigo, cranial tomography (CT) was performed and we also examined the images of the diffusion magnetic resonance imaging (DW-MRI) if we did not observe any pathological finding in CT. In our study, exclusion criteria are shown in Table 1.

**Table 1.** Exclusion criteria.

1	Not give his/her informed consent
2	Not want to share medical information due to any reason
3	Previous cerebrovascular event (CVE) or trans-ischemic attack (TIA)
4	Below the age of 18 years
5	Hypertensive encephalopathy
6	Vascular dementia
7	Liver insufficiency
8	Chronic kidney failure
9	Pregnant or breastfeeding
10	Trauma
11	Optic nerve trauma
12	Optic neuritis
13	Optic nerve arachnoid cyst and mass lesion in the orbita/cavernous sinus

A trained emergency medicine specialist carried out 7.5 MHz linear probe measurement, after the application of a thin layer of a gel on both eyes of the patients, who were in the supine position. The diameter of the optic nerve sheath was evaluated with transverse and sagittal measurements at the 3 mm beyond the posterior of the eye globe. The mean value of the ONSD was calculated as the mean value of the transverse and sagittal ONSD measurements. All images were confirmed by a blinded emergency medicine specialist and the groups were compared.

**Table 2.** The comparison of demographic characteristics of patients and the control group.

Parameters		Central (n=22)	Peripheral (n=91)	<i>p</i>
Age (year), Median (IQR)		60.5 (35)	43 (15)	0.001*
Gender, n (%)	Male	11 (50)	39 (42.9)	0.545**
	Female	11 (50)	52 (57.1)	
Duration (hours), Median (IQR)		16 (19)	4 (6)	<0.001*
Vertigo attack in the medical history, n (%)		3 (13.6)	19 (20.6)	0.559***
Symptoms, n (%)	Headache	8 (36.4)	14 (15.4)	0.036***
	Sudden Onset	11 (50.0)	77 (84.6)	0.001***
	Nausea/vomiting	13 (59.1)	62 (68.1)	0.421**
	Hearing impairment	4 (18.2)	30 (33.0)	0.175**
	The feeling of fullness in the ear	0	25 (27.5)	0.003**
	Tinnitus	2 (9.1)	38 (41.8)	0.004**
	Balance disorder	10 (45.5)	21 (23.1)	0.035**
Nystagmus, n (%)	None	7 (31.8)	41 (45.1)	<0.001*
	Horizontal	6 (27.3)	50 (54.9)	
	Vertical/rotatory	9 (40.9)		
Imaging, n (%)	Restriction in D-MRI	8 (36.4)		
	Cerebellar infarction in CT	5 (22.7)		
	Lacunar infarction in CT	2 (9.1)		
	Bleeding in CT	1 (4.5)		
	No special finding in CT and MRI	6 (27.3)		
Vital Signs, Median (IQR)	Systolic blood pressure (mmHg)	134.5 (19)	133 (38)	0.488*
	Diastolic blood pressure (mmHg)	81 (12)	81 (16)	0.881*
	Pulse (beats/min)	82.5 (17)	75 (22)	0.189*
Comorbidity, n (%)	Comorbidity	13 (59.1)	33 (36.3)	0.050**
	Hypertension	6 (27.3)	17 (18.7)	0.384***
	Diabetes mellitus	3 (13.6)	14 (15.4)	0.837***
	Previous CerebroVascularAccident	3 (13.6)	8 (8.8)	0.491**
	Other	4 (18.2)	4 (4.4)	0.045***

\* Mann-Whitney U, \*\* Pearson's Chi-square test, \*\*\* Fisher's Exact test, n: number of patients, CT: cranial tomography, MRI: magnetic resonance imaging, D-MRI: diffusion magnetic resonance imaging.

### Statistical analysis

The data were uploaded to a computer and analyzed with SPSS (Statistical Package for Social Sciences) Windows v22.0 software package. The descriptive statistics were expressed in numbers (n) and in percentages. The distribution of the continuous data was analyzed with the Kolmogorov-Smirnov test. Median and interquartile range (IQR) were used for the evaluation of the quantitative data. Mann-Whitney U test was used for the comparison of the continuous data and Chi-square test and Fisher's Exact test were used for the comparison of the categorical data. ROC curves were drawn in order to test the role of ONSD in the differential diagnosis of central and peripheral vertigo. The sensitivity and specificity were calculated for central vertigo. The results were evaluated in a confidence interval of 95 % and at a significance level of  $p < 0.05$ .

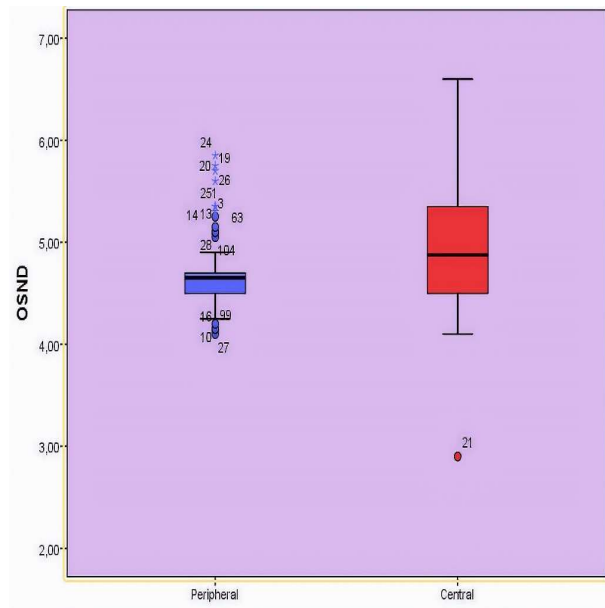
### Results

The median age of our patients was 43 years (IQR: 17) and 44.2 % of them were males. 22 patients (19.5%) had central and 91 patients (80.5%) peripheral vertigo. The mean age of the patients with central vertigo was higher and the duration of vertigo was longer compared to the patients with peripheral vertigo ( $p < 0.05$ ).

The frequency of vertigo attacks was comparable between the genders and the groups ( $p > 0.05$ ). Although headache and balance problems were more common in the central vertigo group, sudden onset, feeling of fullness in the ear and tinnitus were the prominent characteristics of peripheral vertigo ( $p < 0.05$ ). 57.5% of the patients had nystagmus and vertical/rotatory nystagmus was significantly more common in the central vertigo group ( $p < 0.05$ ). Regarding the imaging examination of the central vertigo patients, the restriction

was most commonly encountered in the D-MRI examination (36.4 %). There was no difference between the groups in terms of vital parameters and comorbidity ( $p > 0.05$ ) (Table 2).

In our study, the median ONSD was 4.88 mm (IQR=0.86) in patients with central vertigo and 4.65 mm (IQR=0.20) in patients with peripheral vertigo. The median of ONSD in patients with central vertigo was significantly higher ( $p = 0.030$ ) (Figure 1).

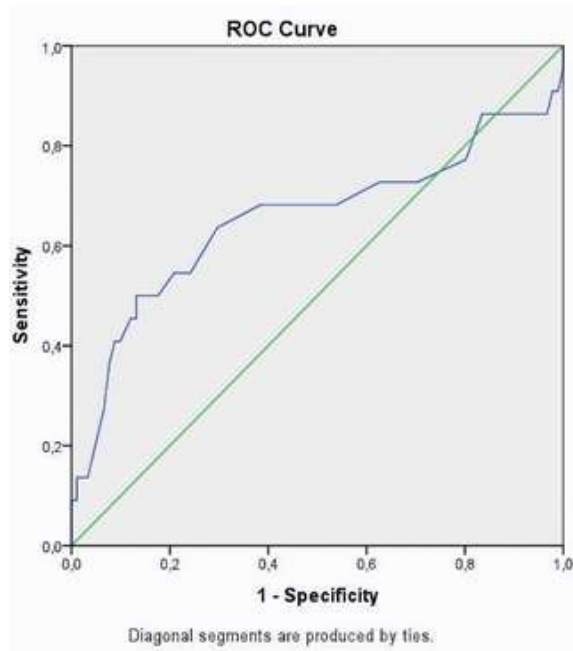


**Figure 1.** The comparison of the groups for ONSD.

ROC curves were drawn in order to test the role of ONSD in the differential diagnosis of central and peripheral vertigo. The area under the curve was 0.654 (95 % CI=0.498-0.810) and the sensitivity and specificity for the cut-off value of 4.65 mm were 68.2 % and 61.5 % respectively (Figure 2).

### Discussion

We found no study in the literature focused on the ONSD measurements for the differential diagnosis of the central and peripheral vertigo. Nevertheless, it has been demonstrated that ONSD was increased in strokes and traumatic



**Figure 2.** Diagnostic ROC for ONSD.

hemorrhages [6, 10-12]. The impairment of the cerebral blood perfusion due to the hypoxia, bleeding and edema and the increase of ICP due to the impairment in the CSF circulation may lead to the dilatation of ONSD [13,14]. In our study, the ONSD was significantly larger in the patients with central vertigo. We believe that this finding depended on the increase of ICP due to the emergence of edema and the deterioration of the CSF circulation.

Batur reported in his thesis study that the sensitivity and specificity of the US in ICP was 95.7 % and 100 % respectively [15]. In patients with ischemia, the sensitivity and specificity were 76-80 % and 84-86 % respectively [6,16]. In the patients with intracranial bleeding, the sensitivity and specificity were 74-100 % and 72.7-100 % respectively [17,18]. In our study, regarding central vertigo, for a cut-off value of 4.65 mm, the sensitivity and specificity were 68.2 % and 61.5 % respectively. The specificity and sensitivity levels were lower than the previous studies and it might be related that the present disorders like ischemia/bleeding did not

severely affect the CSF circulation. In addition, certain disorders, which did not increase ICP (vestibular migraine, multiple sclerosis, epileptic migraine etc.) but cause central vertigo, might decrease the sensitivity. The misdiagnosis of some central vertigo as peripheral vertigo might be the most important reason for the low level of the specificity.

The optic nerve is considered as an extension of the brain tissue, as it originates directly from the brain tissue, is protected by the same sheaths (dura mater, arachnoid and pia mater) as the brain and has Schwann's sheath in its structure [19]. ICP, which is common in the intracranial events, increases the mortality and morbidity in addition to the secondary injuries [17,20]. Xue et al. suggested that 17 % of all vertigo cases depended on central causes [3]. However, Hain and Yacovino stated that the rate of the central vertigo is below 5 % [21]. Branch and Barton reported that the rate of central vertigo was 10 % and added that the rate of central vertigo might increase up to 20 % with aging [2]. In our study, the rate of central vertigo was 19.5 %, which was higher than the reported rates in the literature. The relatively older patient population of our study may be the main reason for this result.

Tinetti et al. [22] and Lin et al. [23] reported that the rate of dizziness might be increased up to 38 % in the elderly patients. Gönüllü and Aygün reported in their study that vertigo was more common in women and central vertigo was more common in elderly patients [24]. They stated that this might be a result of the impairment of the nerve structures, of the increase in depression, and cardiovascular disease rates and of the side effects of the medication. It has also been reported that elderly patients are more likely to apply with central vertigo [2]. In our study, we determined that central vertigo was more common in older



male patients. This finding might be related to the comorbidities, which were increased with aging; to the impaired metabolism and to the decline of the ischemic events in women due to the estrogen. We also believe that the previous strokes have a special place in the etiology of central vertigo.

Nystagmus depends on the continuous stimuli to the optic nerve, which emerge to preserve the accommodation with the help of the chemical and electrical mediators, which were released in the brain as a response to the new condition caused by the dizziness [18]. Studies have demonstrated that sudden onset, horizontal nystagmus and hearing symptoms are prominent in peripheral vertigo and slow onset, vertical/rotatory nystagmus, headache and balance disorders are at the forefront in central vertigo [25,26]. In our study, we determined that slow onset, headache, balance problems and vertical/rotatory nystagmus were more common in the patients with central vertigo and sudden onset, feeling of fullness in the ear, and tinnitus and horizontal nystagmus were more common in patients with peripheral vertigo. We believe that in central vertigo, central events (cerebellar infarction, vestibular migraine, intracranial hemorrhage etc.) cause headache and balance problems due to the emergence of the irritation and the impact on the balance system. On the other hand, symptoms related to the ear are more common in peripheral vertigo, as it is mainly caused by the vestibular dysfunction. It may be suggested that the symptoms progress slowly depending on the penumbra, which enlarges with time in the ischemic events.

Xue et al. investigated 2481 patients with central vertigo and reported that central vertigo emerged due to the cerebrovascular disorders (59 %), vestibular migraine (21.6 %), vestibular paroxysmia (13.5 %), degenerative disorders (3

%), sensorineural hearing loss (2 %) and multiple sclerosis/optic neuromyelitis (0.9 %) [3]. Riberio et al. stated that besides ischemia and bleeding, central vertigo was also caused by certain conditions like tumors, infections and anatomical disorders [27]. In our study, the most common finding was infarction and we did not observe any pathological finding in 27.3 % of the patients. We believe that the most common cause of the central vertigo is ischemia, which was also a finding in our study. In addition, we also believe that 27.3 % of the patients remained undiagnosed, as some ischemia and small bleeding areas could not be detected with the imaging methods and other imaging methods were needed for certain diagnosis except CT and DW-MRI.

Berkiten et al. reported in their study that high blood pressure might provoke vertigo [25]. In some studies, it was demonstrated that in certain comorbidities, in which stroke was common, the rate of the stroke-related vertigo was increased [13,14,28]. It is also known that some drugs trigger vertigo [2,3]. In our study, we did not detect any correlation between the comorbidities and the vital parameters. We believe that this finding depended on the fact that the factors related to the comorbidities were found in both central and peripheral vertigo. We also believe that it was increased due to itself in central vertigo and due to the elevated stress in peripheral vertigo and therefore there was no difference between the groups.

The most important limitation of our study is a single center study, and it was carried out on a small population. Our results must be supported by multi-center studies.

### **Conclusion**

We conclude that the ONSD measurement may be used to support the differential diagnosis of the central and peripheral vertigo. Further

studies with larger subject size are needed on this topic.

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## Responses of salivary cortisol levels and sedation score to oral hydroxyzine premedication in children undergoing outpatient surgery

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### ABSTRACT

**Aim:** To evaluate the sedation score response and salivary cortisol levels (SC) to premedication with sedative hydroxyzine in children with outpatient surgery and the relationships between the two.

**Methods:** Eighty-seven ASA 1 classified patients (American Society of Anesthesiologists Classification 1, normal healthy patients), aged 4-13 years, were randomly and prospectively allocated into the study. Children having outpatient surgery (e.g. inguinal/abdominal surgery, circumcision) either did not have a premedication or received oral hydroxyzine (2 h before the surgery) as a sedative drug. All patients were evaluated for the level of sedation by Ramsay sedation score [RSS, from 1 (awake, anxious, restless or both) to 6 (asleep, exhibits no response)] by an independent anesthesiologist. Salivary samples taken during the assessment of sedation score were analyzed for cortisol levels.

**Results:** SC increased significantly by increasing age ( $r=0.447$ ;  $p<0.001$ ). Premedication with hydroxyzine produced higher sedation scores ( $1.73$  vs  $1.46$ ,  $p=0.014$ ) and patients with higher sedation scores had lower SC ( $p<0.01$ ). Circumcised children had similar SC to hernia/inguinal surgery ( $p>0.05$ ).

**Conclusion:** The data suggest that salivary cortisol increases by increased age and provide evidence that sedation is associated with suppressed cortisol levels. Moreover, different types of surgery appear to be perceived as similar threats by the children.

**Keywords:** Outpatient surgery, conscious sedation, sedation score, salivary cortisol levels, child.

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### Introduction

Undergoing a surgical operation is a stressful event for most of the people but it has much

pronounced effects in pediatric patients [1]. Children are more sensitive to being separated from their parents and unfamiliar surgical environment poses more stress on them, resulting in intense fear, stress and anxiety. In order to overcome this problem, presence of parents before anesthesia has been preferred by the patients, parents and surgeons. However, some studies showed that this method reduced anxiety and improved patient cooperation but

the others expressed their concerns as it caused negative behaviors in children (e.g. exaggerated response to stress) and parents (e.g. emotional outcomes) [2,3].

Sedative premedication might be the other way to alleviate fear, stress and anxiety in children undergoing surgery. The major objectives of premedication are to decrease the stress response with preservation of hemodynamic parameters, to facilitate anesthesia induction and to produce amnesia [1,3]. Commonly used premedications include benzodiazepines, opioids, phenothiazines, barbiturates, and antihistaminics (diphenhydramine, hydroxyzine) [4]. In general, most of them are long acting, physiologically more disturbing and requires parenteral administration [1]. However, availability of oral drugs for premedication has increased their usage, especially in children. Hydroxyzine is one of those orally used drugs and has anxiolytic, antihistaminic, antispasmodic, antiemetic, and secretion lowering effects with minimal respiratory and circulatory changes [5,6]. Level of sedation before surgery, whether induced by premedication or not, is crucial for successful accomplishment of anesthesia and surgery.

Fear, anxiety and stress activate the hypothalamo-pituitary-adrenal (HPA) axis and increases cortisol levels in blood circulation [7]. Therefore, its measurement in blood might provide valuable information about the stress-induced activity of this system. However, blood cortisol is not preferred as it reflects total cortisol (free plus bound cortisol) and as blood sampling by a needle itself activates stress axis. Measurement of cortisol in saliva has taken precedence over blood cortisol, not only due to its non-invasive sampling but also due to its representation of free, or active, cortisol [8]. Also, routine free cortisol measurement in blood is very uncommon and requires labor-

intensive analyses and calculations based on assumptions. Moreover, salivary cortisol is preferable as cortisol freely passes from blood to saliva without being affected by salivary flow rate.

Studies have shown that sedative premedication before anesthesia facilitates patient cooperation, separation from the parents, and eases anesthesia induction [3]. We postulate that this might be due to suppression of stress axis, and therefore, might be associated with reduced salivary cortisol levels. To test this hypothesis, the sedation scores of children undergoing outpatient surgery (whether premedicated or not) were evaluated and then compared with salivary cortisol levels.

### **Materials and Methods**

The study was carried out following ethical approval (*Date: 2016; Decision number: 8-57*) by the local ethics committee (Erzurum Regional Education and Research Hospital Ethics Committee). Written informed consents were obtained from the parents of all patients. The study was conducted prospectively in the Erzurum Regional Education and Research Hospital between October 2016 and June 2017. In the pediatric surgery clinic, 87 patients (4 to 13-year-old) with ASA I scores (American Society of Anesthesiologists) undergoing elective outpatient surgery were included in the study. Patients with any chronic disease, drug use or allergic history were excluded from the study.

The patients either did not receive any premedication or received hydroxyzine groups. The latter group of patients received oral hydroxyzine HCl (Atarax syrup, 2 mg / ml, 200 ml, UCB Pharma, Turkey) 2 hours before the surgery at a 1 ml/kg doses in 10 ml of injectable water. None of the patients were given psychotherapy to reduce anxiety or fear. All the

patients were taken to the operation room and the sedation scores were assessed by using Ramsay Sedation Score scale (RSS, *Score 1: Awake, anxious, restless or both; 2: Awake, cooperative, oriented and tranquil; 3: Responds to commands only; 4: A brisk response to a light glabellar tap; 5: A sluggish response to a light glabellar tap; 6: No response, asleep*) by an anesthesiologist who was unaware of the premedication. Then, the saliva samples were taken from the patients by the surgeon who was going to perform the operation.

Children taken into the surgery should normally wake up early in the morning (presumably at 07.00 am) in order to finish their pre-surgery official procedures in the hospital between 08:30-09:00 am. They had their surgery between 10:00-11:30 am. Although, salivary cortisol have a diurnal rhythm, Petrowski et al. [9] has shown that the rise associated with cortisol awakening response (CAR) settle back to a relatively stable level approximately 3 hours post-awakening. Therefore, the time points for cortisol samplings in the current study coincide with a relatively stable cortisol level. The cortisol awakening response (CAR) is normally associated with a peak cortisol levels between 15-45 min post-awakenings, followed by a decline afterwards. Saliva samples (0.5-1.0 ml) were taken with the help of Pasteur pipette into the micro centrifuge vials. After taking the sample, the tube was sealed, labeled and stored at -20 °C until analysis. As a strict routine before the surgery, the children were not permitted to eat or drink before the surgery. Salivary cortisol levels were analyzed by a validated ELISA (enzyme-linked immunosorbent assay) method reported by Ozgocer et al (10).

Of 87 cases, 41 did not receive premedication and 46 received hydroxyzine. Of the children

with premedication, 89.1% were male (n=41) and this ratio was 100% in the children who had no premedication (n=41). Number of female patients was 5 and they were all among premedication patients. The mean age was 8.0 (range 4 to 12 years old) in the children who had premedication while it was 8.1 (range 4 to 13 years old) in the children who did not have it. All patients underwent a outpatient surgery including inguinal hernia repair, circumcision, hydrocele etc., and were discharged on the same day postoperatively. The demographic characteristics of the patients are shown in Table 1.

For the surgery type, only the patients having either inguinal/hernia surgery (n=35) or circumcision (n=45) were compared as there was sufficient number of patients under these grouping. Patients (n=7) who could not be included in either groups (such as anal fissure, hypospadias etc.) were within premedication group and were not included in the inguinal/hernia surgery vs. circumcision comparisons. Additionally, patients with multiple operations, such as hernia plus circumcision (n=4), were included in the inguinal/hernia surgery group.

### **Statistical analysis**

Data were analyzed by using Minitab 18 statistical package (PA, USA). Distribution of the data was analyzed by using Anderson-Darling test. Cortisol data did not have a normal distribution and therefore they were converted into log 10 scale. One patient's log 10 cortisol value (male, 7 year-old, and circumcised child) was removed from cortisol analyses following Grubbs' outlier test. Consequently, log transformed data had normal distribution and were used for statistical analyses. The effects of age, surgery type and sedation score on salivary cortisol levels were analyzed separately by t-

test. Effect of gender could not be assessed as the number of females were low (n=5). Effects of premedication on salivary cortisol levels were analyzed by t-test. The effects of sedation score on salivary cortisol levels also were assessed by t-test. This data was re-analyzed by following adjusting for age, gender, premedication and type of surgery in GLM (Generalized linear model) analysis (alone or in combination). Same method was also applied to type of surgery comparison for cortisol levels.

Non-parametric sedation scores were compared by Kruskal-Wallis test. Two patients (2 males, both with inguinal hernia surgery) did not have their RSS records and were not included in RSS analyses. Pearson correlations were carried out to find the relationships between the study parameters. An alpha level less than 0.05 was accepted as statistically significant and all data are presented as mean±SEM unless otherwise stated. Salivary cortisol levels are presented as log 10 transformed values.

## Results

The demographic characteristics of the patients were presented in table 1.

**Table 1.** Demographic characteristics of the patients.

Parameters	No-pre med N=41	Pre med N=46
Age (years)	8.1 (4-13)	8.0 (4-12)
Gender		
Male	41 (100%)	41 (89.1%)
Female	0 (0%)	5 (9.9%)
Surgical procedure*		
Inguinal/hernia surgery	7 (17.1%)	28 (71.8%)
Circumcision	34 (82.9%)	11 (28.2%)

Pre med: Pre medication.

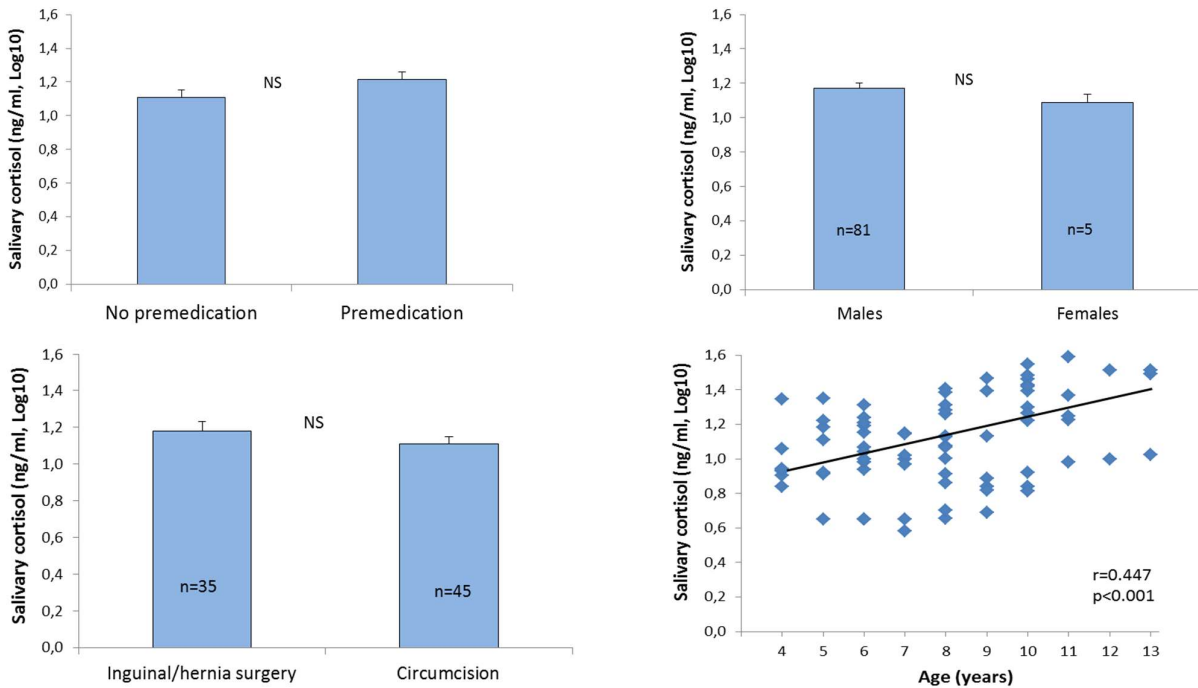
Ramsay sedation score was either 1 or 2 and none of the patients had a sedation score of 3 or more as expected. Premedication caused significantly higher sedation scores (1.73 and 1.46, respectively,  $p<0.05$ ). In the premedication group, sedation score was 2 (awake, calm, watching the environment) in 33 patients (71.7%) but it was score 1 (awake, restless and / or crying) in 11 patients (29.3%). In the other patients without premedication, 19 patients (37.4%) were in score 2 while 22 patients (63.6%) were in score 1 (Table 2). Sedation score did not differ between the genders, between the ages of children or between the type of surgery ( $p>0.05$ ).

**Table 2.** Ramsay sedation scores (RSS) of the patients.

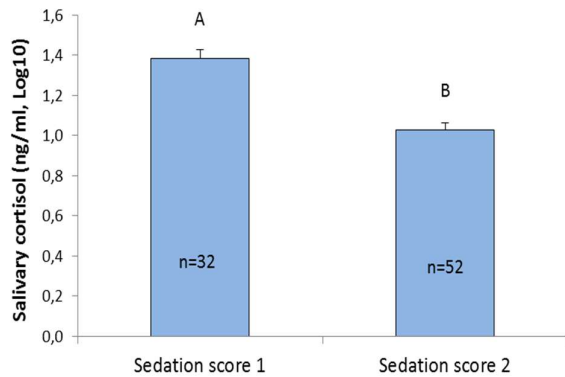
RSS	No-pre med	Pre med	p value
Median (mean)	1 (1.46)	2 (1.73)	<0.05
Min-max	1-2	1-2	
Score 1 (n, %)	22 (53,7)	11 (25,0)	
Score 2 (n, %)	19 (46,3)	33 (75,0)	

Pre med: Pre medication.

Premedication did not affect SC ( $p>0.05$ ) (Figure 1A). Type of the surgery also did not affect SC ( $p>0.05$ ) (Figure 1B). However, the age of the children was significantly, positively and linearly correlated with SC ( $r=0.447$ ;  $p<0.001$ ) (Figure 1C) Children with a Ramsay sedation score (RSS) of 2 had significantly lower SC ( $p<0.001$ ) than that of children who had a RSS of 1 ( $p<0.001$ ) (Figure 2). This trend persisted when the data were adjusted for age, type of surgery, gender and premedication, alone or in combination ( $p<0.001$ ).



**Figure 1.** A. Salivary cortisol (SC) did not differ between patients who had premedication or not ( $p>0.05$ ). B. SC did not also differ between males and females ( $p>0.05$ ). C. Type of surgery did not have an effect on SC ( $p>0.05$ ), B. Age of the children significantly, linearly and positively correlated with SC ( $r=0.447$ ;  $p<0.001$ ). NS, non-significant. Data are presented as log10 mean  $\pm$  S.E.M.



**Figure 2.** Children with higher Ramsay sedation scores (RSS) had lower SC ( $p<0.001$ ). This trend did not change in significance when the data were adjusted for age, type of surgery, premedication and gender in GLM analyses (alone or in combinations). Data are presented as log 10 mean  $\pm$  S.E.M. Different letters denote significant difference at  $p<0.001$ .

**Discussion**

This study evaluated preoperative sedation level and stress axis in children by non-invasive means (salivary cortisol) and provided evidence to support the hypothesis that higher sedation

scores (score 2 vs. score 1), irrespective of premedication use, are associated with suppressed salivary cortisol levels. Features of the patients, sedation scores and salivary cortisol levels are separately discussed below.

This study was randomized prospective study and most of the patients attending to clinic were boys (94.3%). When the patients who were circumcised were excluded (as it is a seasonal event coinciding with the study period), again the vast majority of the patients (88.1%) were boys. This appears to be due to the fact that inguinal hernia or inguinal operations are more common in boys [11].

As expected, RSS was higher in patients who had premedication (a mean score of 1.73 vs. 1.46). Additionally, sedation scores were either 1 (awake, restless and / or crying) or 2 (awake, cooperative, oriented and tranquil) and none of the patients had a score 3 or more. These scores were also expected when drugs like hydroxyzine was used for premedication. Some



patients in premedication group had lower RSS while some of the patients in non-premedication group had higher RSS. Both situations may suggest that sedation itself is likely to be a trait feature but hydroxyzine is effective in inducing sedation in most patients. Moreover, type of the surgical operation, age and gender did not have an effect on sedation score [12].

Salivary cortisol levels represent free cortisol, which is the bioactive fraction of total circulating cortisol levels [13,14]. Therefore, it has been considered more appropriate measure than total blood cortisol and it has been accepted as a useful tool to measure anxiety and stress in a non-invasive manner [15,16]. Likewise, in the current study, salivary samples were taken non-invasively before the surgery to assess the activity of the stress axis, i.e. the hypothalamo-pituitary-adrenal axis (HPA).

The age of the children was one of the most important factor affecting salivary cortisol levels. This is in accordance with the study of Bäumlér et al. [17] who observed a linear increase in awakening cortisol levels in children aged 2-87 month (7.25 year-old). The current study adds that the increase was also evident from 4 to 13 years. Moreover, the current study did not measure awakening response but instead it utilized sample taken later in the day. This sample is expected to have lower cortisol concentration as awakening response is the highest response during the daytime. Even so, age-dependent increase was still evident. Furthermore, all children knew that they were going to have a surgical operation when they gave their saliva samples. From that point of view, it appears that stress reactivity against a surgical operation also increases by age. It might be speculated that awareness about the consequences of a surgical operation may increase by age and this, in turn,

may translate into higher stress reactivity in older children. An alternative explanation might be “maturation of the HPA axis” by increasing age [18]. Altogether, data suggest that maturation of HPA axis or awareness of threats increases by increasing age. Moreover, data in the current study shows that this “maturation” or “reactivity” appears to cover not only the neonatal or early childhood period but also continues until late childhood (early teenage). Our hypothesis was that higher sedation scores would be associated with lower salivary cortisol levels. The data in the current study provides support for this hypothesis and suggest that suppression of HPA axis might be an integral part of sedation. Higher sedation scores (score 2) was not only seen in premedication group but also in control group, suggesting that suppression of HPA axis may be a trait feature like sedation scores. In line with this, Hsu et al. [19] observed no change in cortisol levels in drug-induced sedation group over the control. This suggests that lower salivary cortisol levels in higher sedation scores might be due to personal traits rather than direct effects of sedatives. In the current study, total number of girls was quite low (n=5) and therefore it has not been possible to draw conclusions in terms of effects of gender on salivary cortisol levels. Nevertheless, Lindfors et al [20] who found no difference between girls and boys in their response to perceived stress or in relation to recurrent pain such as headache, stomach ache, back pain etc. Additionally, it seems that circumcision, a relatively simple surgery, evokes similar stress responses observed in hernia/inguinal surgery. This might suggest that both groups of surgeries are perceived as similar threats by the children.

### **Conclusion**

This study suggests that sedation in children appears to be associated with suppression of

salivary cortisol levels. Moreover increased age of the children is associated with increased salivary cortisol levels suggesting that maturation or reactivity of the HPA axis might increase by increasing age.

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**Ethical statement:** *The study was conducted in accordance with the ethical approval of the University Ethics Committee (Date: 2016; Decision number: 8-57).*

#### **Acknowledgment**

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

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## A comprehensive review on rational and effective treatment strategies against an invisible enemy; SARS Cov-2 infection

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### ABSTRACT

The year 2020 is began with the declaration of a pandemic of novel coronavirus, which first occurred by the end of 2019 in Wuhan region of China. The novel virus infection is so called as Covid-19 or SARS Cov-2. The infection rapidly spread all over the world and changed the lives of millions. In this extended review, we aimed to discuss current and possible treatment strategies against SARS Cov-2 infection. Treatment options mentioned here include but not limited to chloroquine/hydroxychloroquine, favipiravir, remdesivir, lopinavir/ritonavir, umifenovir, steroids, cepharanthine, convalescent plasma, anticoagulants and monoclonal antibodies. In conclusion, mainstay of the SARS Cov-2 treatment is general measures such as patient isolation and supportive care. However, encouraging developments are being achieved in terms of discovery of an effective treatment and production of a potent vaccine.

**Keywords:** Covid-19, SARS Cov2, treatment, vaccine.

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### Introduction

By the end of 2019, a new pandemic of coronavirus (Covid-19 or namely, SARS Cov-2) arose from China and rapidly spread to the rest of the world. It is not only an important infectious disease but also a concern of public health emergency [1]. Angiotensin-converting enzyme 2 (ACE2) receptors play important role in the infection since surface spike proteins of the virus bind to these receptors and enter the human cells [2]. Type II alveolar cells in the

lungs express ACE2 receptors, therefore, devastating effects of the infection mainly occur in the lungs [3]. Heart, kidneys, vascular endothelium and intestinal epithelium also express ACE2 which could be the underlying mechanism of multiorgan dysfunction and micro-thrombi formation that caused by SARS Cov-2 [4,5]. Although the SARS Cov-2 infection could be asymptomatic, about 15% of the patients complicate with the severe course of the disease [6]. The infection could begin with flu-like symptoms. Indeed, nearly 2 or 3 of every 4 subjects that have positive RT-PCR throat swab results remain without symptoms and only in 10% of the symptomatic patients develop dyspnea, interstitial pneumonia, ARDS and/or multiorgan dysfunction [7]. Fever,

headache, myalgia, fatigue, rhinorrhea, cough, mild dyspnea, sore throat and conjunctivitis are common symptoms of the disease [8,9], which all seen in other respiratory conditions as well. Rare presentations of the infection include diarrhea, nausea and vomiting [10]. Determining the mortality rate of SARS Cov-2 infection is difficult since it might be an earlier period of the pandemic, however, Jiang et al reported the mortality rate of the disease was 3% [11]. Although this rate is lower than previous coronavirus infections; severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS), which have mortality rate of 10% and 35%, respectively, elderly population and subjects with chronic diseases (i.e. diabetes mellitus, hypertension, heart failure, coronary heart disease) tend to have more serious clinical course and increased risk of death. As of 29th of September in 2020, 33,249,563 people infected with Covid-19 and 1,000,040 of them died in the whole world [12]. More than half of the cases (16,434,186) and deaths (551,313) were reported from North and South America [12]. Unluckily, to date, there is no proven and effective treatment against SARS Cov-2 infection [13]. However, several drugs and attempts to develop a vaccine are on the way.

The purpose of this review is evaluating the current experimental and clinical treatment options of SARS Cov-2 infection, as well as focusing the attempts on developing an effective vaccine for the disease.

### ***Current treatment option of SARS Cov-2***

#### ***Oxygen supplementation***

This is an important step in the management of SARS Cov-2 after obtaining protective measures such as isolation of the patients. A number of subjects with SARS Cov-2 may

develop hypoxemia during the course of the infection. Supplemental oxygen is indicated if the oxygen saturation drop below 93% or in cases with evident respiratory distress [10]. Face mask, nasal cannula or non-invasive ventilation are the routes of oxygen therapy [14]. Prone positioning may be useful in oxygen supplementation [15]. Indeed, most of the patients in critical care may respond well to prone positioning [14,16]. Endotracheal intubation is evitable when the oxygen saturation is not reached to target levels.

#### ***Chloroquine/hydroxychloroquine***

Chloroquine and Hydroxychloroquine are antimalarial drugs which are also used in rheumatologic diseases. They have immunomodulatory effect and were used against SARS and MERS since they reduced viral replication [17,18]. Hydroxychloroquine is useful in the treatment of autoimmune disorders (i.e. rheumatoid arthritis and Sjögren's syndrome) since it decrease the serum levels of inflammatory cytokines [19]. These effects of the drug might be promising in the severe form of SARS Cov-2, which is associated with cytokine storm.

The first human Covid-19 study about the efficacy of chloroquine showed that duration of the symptoms and severity of the pneumonia was reduced in subjects treated with chloroquine [20]. In a following study, authors compared the nasopharyngeal viral clearance on 6<sup>th</sup> day of the subjects received hydroxychloroquine or hydroxychloroquine plus azithromycin to the controls and reported higher clearance rate in subjects received hydroxychloroquine plus azithromycin (100%) than the patients received hydroxychloroquine alone (57%) and the controls (12.5%) [21]. However, the efficacy of these drugs against SARS Cov-2 infection is controversial. Wang

et al. and Colson et al reported that chloroquine was effectively reduced viral replication in an in vitro study [18,22]. Moreover, antibiotic treatment to prevent bacterial infection is lack of evidence [10].

These drugs' antiviral effects are driven by the immunomodulatory effects, by increased cytoplasmic pH and by glycosylation of cellular receptors of the virus [22]. Glycosylation of cellular receptors may prevent viral binding to ACE2 receptors [23,24]. Both chloroquine and hydroxychloroquine act similarly and prevent the membrane fusion by changing the pH of acidic intracellular organelles (endosome, lysosome) [19]. Therefore, authors believe that these agents could be effective in treatment of SARS and SARS Cov-2 infections [18,25].

Sialic acid, which is a monosaccharide required for ligand recognition and is a part of receptors that used as ligand by coronaviruses and orthomyxoviruses [19]. Chloroquine prevents the synthesis of sialic acid by inhibiting the enzyme quinone reductase-2 [19]. This inhibition could be responsible of the broad antiviral effects of chloroquine [19]. Moreover, chloroquine interferes with the virion budding of SARS Cov-2 via inhibition of MAP-kinase molecular crosstalk [18,22].

In vitro studies showed that hydroxychloroquine is superior to chloroquine in inhibition of SARS Cov-2 infection [26,27]. Beside effectiveness, chloroquine also provides an affordable treatment for the infection, since it is inexpensive. A Chinese consensus report that published in February 2020, declared that chloroquine 500 mg twice daily for ten days was recommended for SARS Cov-2 pneumonia [28]. Netherlands control of disease control also recommended treatment with chloroquine, but only for those that necessitate hospitalization, oxygen supplementation or intensive care support [29]. Moreover, an Italian guide by

Italian Society of Infectious and Tropical disease also suggest treatment with chloroquine or hydroxychloroquine for 10 days for patients with SARS Cov-2 infection [30]. In addition, a Korean task force recommended chloroquine or hydroxychloroquine in treatment of moderate and severe SARS Cov-2 cases [31]. Despite both 500 mg twice daily chloroquine and 200 mg twice daily hydroxychloroquine are suggested as effective treatments for SARS Cov-2 [1,32], optimal therapy requires a loading dose before maintaining dose [33].

Side effects of chloroquine include QT interval prolongation in electrocardiography, hematological disorders (anemia, leukopenia, and thrombocytopenia), elevation in liver enzymes, serum electrolyte disturbances, elevation in renal function tests and visual deterioration. QT prolongation is the most important side effect and this side effect may be potentiated by concomitant use of other drugs that increase QT interval, such as, macrolide antibiotics, antiarrhythmic agents, antidepressants, antipsychotics, ondansetron and quinolones. Therefore, close monitorization of the patients regarding hemogram and ECG is advised.

### ***Favipiravir***

Favipiravir is a purine nucleic acid analog that target RNA viruses by potently and competitively inhibiting the RNA-dependent RNA polymerase [34].

Favipiravir is very effective against infections with influenza A, B and C viruses that resistant to oseltamivir [35]. Novel studies reported its efficacy in treatment of SARS Cov-2 infection [36]. In addition, in vitro studies suggested its efficacy against SARS Cov-2 [22,37]. In a study from China, authors reported that duration of viral clearance of SARS Cov-2 was reduced in patients received favipiravir

compared to those subjects received lopinavir/ritonavir [38]. Moreover, in the same study, radiological improvement rate on day 14 was higher and side effect rate was lower in favipiravir group compared to the patients received lopinavir/ritonavir treatment [38]. Therefore, it has been recommended in the management of this infection in China [39] and in Turkey [40]. Despite there are currently no randomized controlled clinical trial with large cohort about the efficacy of favipiravir in the treatment of SARS Cov-2, studies are on the way about this topic [39].

Starting dose of favipiravir is 1600 mg two times on day 1, 600 mg two times a day on 2<sup>nd</sup> to 5<sup>th</sup> days and 600 mg once a day on 6<sup>th</sup> day of the treatment [39]. Adverse effects of favipiravir include increase in serum uric acid levels, elevation in hepatic enzymes and gastrointestinal discomfort [38,41,42].

### ***Remdesivir***

Remdesivir is a nucleotide analogue that promote early termination of the viral RNA transcription by incorporation into genetic material of the virus [10]. Apart from other antiviral agents classified as nucleotide analogues, it has broad antiviral spectrum including Pneumoviridae, Filoviridae, Paramyxoviridae, and Orthocoronavirinae (i.e. coronaviruses that cause SARS and MERS) families [43,44]. In an animal study, Remdesivir improved organ functions and decreased viral load in mice with MERS infection [45]. Moreover, it has evident antiviral effects against SARS and MERS infections [46]. Good responses achieved with Remdesivir in the treatment of some patients with SARS Cov-2 in China, where the outbreak of Covid-19 first started [47]. In a case report from United States, it was reported that Remdesivir was effective in treatment of a

patients with SARS Cov-2 pneumonia [48]. In a recent study, Remdesivir was suggested to be superior to placebo in achieving shorter recovery duration [49]. Furthermore, clinical improvement was noted in 65% of the subjects with severe SARS Cov-2 infection whom received remdesivir treatment [50]. However, a Chinese study reported that Remdesivir was not superior to placebo in terms of duration between drug initiation and clinical improvement [51].

Serious adverse reactions, including rectal hemorrhage, liver toxicity, and nausea and vomiting, have been reported relate to remdesivir [39]. Although these adverse events upraise questions about Remdesivir in treatment of SARS Cov-2, it has great in vitro efficacy against the virus [43,48]. An initial 200 mg/day of Remdesivir which followed 100 mg daily on 2<sup>nd</sup> day and after is advised in treatment of SARS Cov-2 infection [39].

### ***Lopinavir/ritonavir***

Protease inhibitors are drugs that developed for the treatment of human immunodeficiency virus (HIV) infection. New generation protease inhibitor, the combination of lopinavir and ritonavir, also shows its effect through inhibition of viral protease [10]. Papain-like protease and 3C-like protease in coronaviruses are target enzymes for protease inhibitors [52]. Lopinavir/ritonavir was suggested to be useful in SARS infection [53]. Several studies in literature suggested that this combination reduced the viral load in SARS Cov-2 infected subjects [54,55]. However, the effect of lopinavir/ritonavir treatment against SARS Cov-2 is controversial. In a novel study, remdesivir was suggested to be superior to lopinavir/ritonavir and interferon-beta combination in terms of reducing viral load and the improvement of pneumonia in subjects with

MERS [45]. On the other hand, there are studies in literature that reported no benefit of lopinavir/ritonavir combination greater than standard medical care in SARS Cov-2 infection [56].

Authors speculate that serum level of lopinavir/ritonavir could be much lower than the required serum concentration to inhibit SARS Cov-2 replication [57]. Advised dose of the combination is 400mg/100mg twice a day. Sleeping difficulty, nausea, vomiting and diarrhea are reported adverse effects of the lopinavir/ritonavir.

### ***Umifenovir***

Umifenovir is an antiviral agent that has broad antiviral spectrum. The drug prevents viral entry by blockage of the fusion between the virus and the cell membrane. It blocks viral fusion with the target membrane, thus providing viral entry into target cells. Umifenovir is an indole derivate and its antiviral spectrum covers influenza viruses [58]. Therefore, it is approved in both treatment of and prophylaxis for influenza infections [59]. Authors retrospectively analyzed subjects with SARS Cov-2 infection according to hypoxic (mean age 70 years) and non-hypoxic (mean age 37 years) patients and reported that 58.2% of the non-hypoxic and only 33% of hypoxic subjects were treated umifenovir [60]. Moreover, it was concluded in the same study that 33% of subjects received umifenovir were discharged while 19% of the subjects that not received umifenovir discharged from the hospital, in other way to say, despite the mortality rate of the study population was 7.5%, none of the subjects received umifenovir were deceased [60]. It shall be speculated that umifenovir could increase the discharge rate and decrease the mortality rate in SARS Cov-2 infection, nonetheless, age difference between

study groups may confound the results of that study.

Another study with small population compared umifenovir and lopinavir/ritonavir treatment to lopinavir/ritonavir treatment alone in patients with SARS Cov-2 and found that umifenovir and lopinavir/ritonavir combination was superior to lopinavir/ritonavir alone according to viral clearance on seventh day, improvement in thorax imaging, and viral clearance on 14<sup>th</sup> day [61].

Reported side effects of the umifenovir include nausea, diarrhea and elevation in serum bilirubin levels [59]. Larger prospective cohort studies are needed to suggest for or against the use of umifenovir in the treatment of SARS Cov-2 infection.

### ***Steroids***

Steroids (or namely corticosteroids) are immunosuppressive and anti-inflammatory agents with a broad clinical usage in many disorders. Methyl prednisolone and dexamethasone are two frequently prescribed steroids in daily practice. The use of steroids in patients with severe SARS, MERS and influenza infections reported either no improvement or an increase in mortality rates [62-64]. These nonsuggestive evidences did not discourage researchers to study the effects of steroids in the novel coronavirus pandemic. However, there are conflicting study results in literature about treatment of SARS Cov-2 with steroids. The role of steroids investigated in a small population with SARS Cov-2 whom were treated in intensive care unit because of moderate-severe ARDS, and the results of the study revealed that steroids did not decreased mortality despite hypoxia was improved in the subjects [65]. Similarly, addition of the steroids in treatment regimen reduced the fever but did not decreased the mortality, nor reduced the



resolution duration of pneumonia, nor decreased the length of hospitalization of SARS Cov-2 patients in a meta-analysis [66]. Moreover, low dose of steroids in short course to prevent cytokine storm in SARS Cov-2 patients with disease progression did not alter the clinical outcome of the cases [67]. In contrast, Lee et al proposed early use of steroids in treatment of SARS Cov-2, since it could be difficult to reverse advanced ARDS in these subjects [68]. Indeed, authors reported that short term dexamethasone improved the outcome of the patients whom infected with novel coronavirus [69].

These evidences bring a question in mind whether steroids should be given in an earlier phase of the SARS Cov-2 infection instead of delaying to the advanced stage. The results of prospective studies on the way might answer this question successfully.

### ***Cepharanthine***

Cepharanthine is an alkaloid agent that used for alopecia in Japan for nearly 70 years [70]. Indications of the drug also include exudative middle-ear catarrh [70], leukopenia due to radiation [71], viper and other venomous snake bites [70], alopecia areata [72] and immune thrombocytopenic purpura [73]. It has also been effective against other coronaviruses that cause mild infection in human [74].

It has also been suggested that cepharanthine reduces nitric oxide production in macrophages and prevent cell death in septic shock [75]. Besides nitric oxide production, it decrease lipid peroxidation, reduces nuclear factor-kappa B activity, and inhibit cyclooxygenase pathway, thus, provide anti-inflammatory effects and improvement in vascular endothelium [76]. Cepharanthine decreases the serum levels inflammatory cytokines which include interleukin-1 beta, tumor necrosis

factor alpha and interleukin-6, the inflammatory predictor that to treat SARS Cov-2 related cytokine storm [77]. Replication of human immunodeficiency virus is shown to be inhibited by cepharanthine more than 2 decades ago [78]. Moreover, its use against SARS Cov-2 and other viral infections is suggested by anti-inflammatory, immuno-modulating and anti-oxidative features of that phytomedicine [70,72]. Indeed cepharanthine was suggested to be an effective treatment option in SARS in 2005 [79].

Cepharanthine has both effects in pre and post entry stages of the SARS Cov-2 infection. It reduces viral RNA in post entry phase [80]. In addition viral replication is also inhibited by cepharanthine [81]. Moreover, authors showed that cepharanthine has inhibitor effect on S glycoprotein of SARS Cov-2 virus, which is essential for the virus entry to human cells [82]. Its utility in novel pandemic is also being investigated since SARSCov-2 has great homology with SARS in terms of genomic features. An animal study revealed that it was successful in treatment of SARS Cov-2 infection [80]. It has been reported in an unpublished cell culture model study that it has great efficacy against SARS Cov-2 [83]. Cepharanthine was proposed as a drug that has antiviral activity against SARSCov-2 [84], however, clinical human studies in randomized controlled trial form are required to demonstrate its efficacy in the treatment of SARS Cov-2 patients.

### ***Convalescent plasma***

Plasma of a patient who healed from an infection contains antibodies against a certain microorganism. Infusion of this plasma to another person suffering of the same infection is called as convalescent plasma therapy, which is a form of passive immunotherapy [59].

Convalescent plasma therapy was used in previous viral infections, such as, influenza, SARS and Ebola [85,86]. Authors speculate that this treatment option might reduce mortality in severe viral respiratory infections, however, the majority of the studies suggesting convalescent plasma treatment were low quality reports without a control group [87]. Plasma of the patients that recovered from SARS Cov-2 is being used in the treatment of the disease. The FDA was approved its emergency investigational use in subjects with severe SARS Cov-2 infection [88]. In a case series, convalescent plasma found to improve clinical symptoms and to promote resolution of ARDS in SARS Cov-2 patients [86]. A case report including two patients from Korea revealed that clinical improvement and radiological resolution were achieved with plasma therapy [89]. In a study with small cohort, it was suggested that convalescent plasma was improved outcome of the patients with SARS Cov-2 [90]. These data suggest that convalescent plasma treatment may be useful in SARS Cov-2 infection; however, studies with larger population are needed to confirm its effectiveness in the treatment of the disease. Adverse reactions related to plasma therapy include transmission of disease, circulatory overload, allergic reactions and transfusion associated lung injury [91].

### ***Antibiotics***

During SARS Cov-2 infection, the rate of co-infections with other microorganisms could be as high as 50% [39]. Influenza A virus is the most commonly reported virus that cause co-infection [92]. Other causes of co-infection include various bacteria (including *M. pneumoniae*), fungi (*Candida* spp.), and viral pathogens (other coronaviruses, rhinovirus, human immunodeficiency virus) [39]. At the

early stages of the pandemic, antibacterial and antiviral agents against influenza were frequently added in the treatment regimens against SARS Cov-2. Moreover, patients that hospitalized for a week or longer may require effective antibacterial therapy against pneumococci, *S. aureus*, *K. pneumoniae*, *Pseudomonas* spp. and *Acinetobacter Baumannii* [93,94].

Azithromycin is a macrolide antibacterial agent which prevents concomitant bacterial infections during viral respiratory infections [95]. Moreover, it has antiviral effects in vitro, suggested by Madrid et al, in 2015 [96]. During SARS Cov-2 pandemic, it was used along with hydroxychloroquine in treatment of patients in severe condition and triggered good clinical outcome [21]. Azithromycin may increase liver enzymes and combination with hydroxychloroquine warrant more attention on prolongation of QT interval in electrocardiogram.

Teicoplanin, a glycopeptide antibiotic, could be a promising antiviral agent as well. Cathepsins L and B in human cells are responsible of the release of viral genome into the cytoplasm by cleaving viral proteins [97]. Teicoplanin inhibits the activities of both cathepsins L and B, specifically [24]. Indeed, it prevents viral entry in Ebola, SARS and MERs infections [98]. These data suggest that viral infections that require cathepsin L to enter human cells may benefit from treatment with teicoplanin and its derivatives: dalbavancin, telavancin, and oritavancin [94]. Nevertheless, we think that randomized trials on teicoplanin treatment in patients with SARS Cov-2 infection are needed to confirm its effectiveness against the virus.

### ***Monoclonal antibodies***

Monoclonal antibodies are developed and used against viral infections such as influenza, SARS

and MERS infections [43,99,100]. Exacerbation of rheumatoid arthritis is being treated with tocilizumab, a monoclonal antibody that prevents binding of interleukin-6 to its receptors [94]. It can also be effective in alleviating cytokine storm in patients with severe SARS Cov-2 infection. Therefore, its effectiveness in the treatment of the infection is being studied. In a study from China, authors suggested that tocilizumab was effective in treatment of cytokine storm during severe SARS Cov-2 infection [101]. Since cytokine storm occur in a significant amount of subjects with severe infection, tocilizumab could prevent this cytokine related damage and death by inhibiting the effects of interleukin-6 [102]. Nevertheless, evidence will accumulate about the role of tocilizumab in the treatment of the infection by growing experience on its use against SARS Cov-2.

#### ***Anticoagulant treatment***

Besides sepsis induced coagulopathy, disseminated intravascular coagulation and venous thromboembolism (driven by prolonged inactivity) could occur as complications in all critically ill subjects, thrombotic complications are especially predisposed by severe SARS Cov-2 infection [59]. Coagulation system is triggered by inflammation and infection during SARS Cov-2 infection. Over activation of the coagulant factors in blood may result in ischemic events, thrombi formation and disseminated intravascular coagulation [103]. Indeed, the rate of thrombotic complications (myocardial infarction, ischemic stroke and arterial embolism) was reported as high as 31% in SARS Cov-2 subjects whom treated in intensive care unit [104]. A similar study from China reported 25% of venous thrombosis in cases with severe SARS Cov-2 [105]. Anticoagulation therapy is advised in earlier

periods of the SARSCov-2 infection especially when the d-dimer levels are higher than the four times of upper limit of normal range [10]. Other markers of pro-coagulant state in SARS cov-2 infection are increased fibrin degradation product levels, inflammatory predictors, and prolonged prothrombin time and activated partial thromboplastin time levels, which all related with the increased risk of mortality [106]. On the other hand, it is useful in patients with severe condition, too. A study by Tang et al suggested that heparin (mostly low molecular weight heparin) treatment was related with better prognosis and reduced mortality in severe SARS Cov-2 cases [107]. In contrast, both the durations of hospitalization and viral clearance of severe SARS Cov-2 patients received low molecular weight heparin were not different from the subjects that not received anticoagulant therapy [108]. Nevertheless, World Health Organization recommends heparin or low molecular weight heparin as a prophylactic treatment against venous thromboembolism in severe SARS Cov-2 patients [109]. Furthermore, not only intensive care population but also all hospitalized SARS Cov-2 subjects are suggested to be prescribed low molecular weight heparin if it is not contra indicated (i.e. active bleeding, a platelet count lower than 25000 per microliter) [110].

#### ***Other possible treatment options***

Hepatitis C virus, several hemorrhagic fever viruses and respiratory syncytial virus are being treated with ribavirin, a nucleotide analogue. It has previously been used against SARS infection [111]. It was also proposed as an effective treatment option against SARS Cov-2 in a study from Saudi Arabia [112]. Moreover, authors supposed that ribavirin, as a direct anti-viral agent, could be used against SARS Cov-2 infection [113]. Reduction in blood hemoglobin

is the most important side effect of ribavirin which may limit its use since that is harmful in subjects with respiratory distress [44].

Interferon 1 beta is used in the treatment of MERS infection [111]. However, its effect against SARS was uncertain [52]. In a multicenter, prospective, open-labeled, randomized, phase 2 trial, 81 subjects enrolled to the treatment group that received combination of lopinavir 400 mg and ritonavir 100 mg twice daily, ribavirin 400 mg twice daily, and three doses of 8 million IU of interferon 1 beta on alternate days for 14-days while 41 subjects received lopinavir 400 mg and ritonavir 100 mg twice daily for 14 days and authors concluded that combination of lopinavir /ritonavir plus ribavirin plus interferon 1 beta treatment was superior to lopinavir/ritonavir treatment alone in shortening viral shedding duration, in reducing hospitalization time and in alleviating of the symptoms in mild to moderate SARS Cov-2 cases [114].

Melatonin has anti-inflammatory, anti-cancer and anti-oxidative properties. Acute lung injury and ARDS is prevented by melatonin [115]. Since it has high safety profile [116] and it reduces the risk of cytokine storm [117], growing data suggest that melatonin could be beneficial in treating viral infections, as well as SARS Cov-2 [115]. Authors found that increased activity of ACE2 might decrease the severity of the infection with respiratory syncytial virus [118]. On the other hand, statins improve endothelial functions which was deteriorated by viral infection, therefore, a combination with angiotensin receptor blocker and statin may enhance recovery of endothelium and facilitate healing of the patients own [119,120].

Nitazoxanide is an antiparasitic and antiviral drug with broad spectrum which is converted to

its active form; tizoxanide following oral intake [121]. Five day course of treatment of influenza with nitazoxanide 600 mg twice daily has been suggested to be effective in relieving symptoms with mild adverse events [122]. Interferons alpha and beta production are upregulated by nitazoxanide. Therefore, its activity against MERS and other coronaviruses were tested and found to be effective in vitro [123]. Promising results of the drug in other viral infections drive a proposal that nitazoxanide could be used in early SARSCov-2 infection in adjunctive to azithromycin [124]. Moreover, authors compare the efficacy of hydroxychloroquine and hydroxychloroquine plus nitazoxanide in patients with SARS Cov-2 and expect better outcome in subjects received combination therapy [125]. Although it is a promising treatment alternative in novel coronavirus infection, controlled trials that suggest its efficacy in management of the disease is still lacking. Studies in recent years revealed the antiviral effects of ivermectin. It is an antimicrobial agent against parasitic infections, however, it has also broad range of antiviral effects. Ivermectin has been shown to inhibit replication of human immunodeficiency virus [126]. Subsequently, it was reported that ivermectin ameliorate the infections with influenza, West Nile, pseudorabies and dengue viruses [126,127]. In contrast, it has shown to be ineffective against Zika virus [128]. Caly et al showed in an in vitro study that ivermectin was potently inhibit the replication of SARS Cov-2 [127]. These encouraging results enabled the authors propose ivermectin as a promising candidate of SARS Cov-2 treatment [129]. On the other hand, randomized controlled trials should suggest the efficacy of the drug in order to recommend ivermectin evidently in the treatment of novel coronavirus pandemic.

### ***Futuristic perspective***

It is speculated that inflammation and tissues damage driven by ARDS might be ameliorated with mesenchymal stromal cells [130]. The interaction between viral proteins and ACE2 might be targeted in following research to develop effective treatment options [131,132]. Isolation and amplification of cytotoxic T lymphocytes against SARS Cov-2 would be beneficial in the treatment of the disease [133]. T lymphocyte mediated inflammatory pathway would be stimulated by canakinumab or roflumilast [134,135]. This list may be prolonged as the knowledge about the SARS Cov-2 virus grows.

### ***Vaccines against SARSCov-2: Will full eradication be possible?***

Effective prophylactic strategy in disease control and prevention of new cases could be achieved with the development of a successful vaccine, therefore, nearly 100 vaccine research is ongoing worldwide [136].

There have been 115 vaccine candidates worldwide as of the first half of April, 2020 [137]. As of 25 August 2020, 31 vaccines are reached the level of clinical evaluation and phase 3 trials have been initiated for six of the vaccine candidates [138]. These 6 vaccine candidates are being developed by University of Oxford/AstraZeneca, Sinovac, Wuhan Institute of Biological Products/Sinopharm, Beijing Institute of Biological Products/Sinopharm, Moderna/NIAID, and BioNTech/Fosun Pharma/Pfizer [138].

Technologies used in the development vaccine against SARS Cov-2 include DNA, RNA, inactivated, live attenuated, non-replicating vector, protein subunit and replicating viral vector [139]. The first vaccine candidates are expected to be in clinical use by the end of 2020 or in 2021. This accelerated vaccine

development race has some risks. If a vaccine stimulate production of antibodies against virus that not neutralize its infectivity, additional consequences may occur via increased viral replication or immune complex formation which accumulate in the tissues and attract inflammatory mediators and trigger complement system [140].

The first and the only confirmed vaccine by state health authorities against SARS Cov-2 is the Sputnik V vaccine of Russian researchers. The vaccine registered in 11th of August in 2020 and developed by Gamaley Epidemiology and Microbiology National Research Center in Russian Federation. Despite public opinion is diverse and trust to the vaccine is distinct in the world, results of the vaccine on volunteers are eagerly and excitedly being awaited.

There are currently over 169 COVID-19 vaccine candidates under development, with 26 of these in the human trial phase [141]. Of those, the phase 3 trial of the vaccine developed by Oxford University has been interrupted due to transverse myelitis, which developed in two of the volunteers, then the trial resumed by the developers [142].

Sputnik V SARS Cov-2 vaccine from Russia, Sinovac's Covid-19 vaccine from China, BioNTech and Pfizer's Covid-19 vaccine from Germany are some of the other vaccine candidates with ongoing phase III trials.

### ***Novel developments in the subject***

The U.S. Food and Drug Administration (FDA) decided to broad the scope of emergency use authorization of remdesivir to include treatment of all adult and pediatric SARS Cov-2 patients in hospital era regardless of the severity of the disease and both in confirmed and suspected cases [143]. Moreover, the Solidarity trial which was established by WHO, discontinued

the hydroxychloroquine and lopinavir/ritonavir arms due to little or no reduction in the mortality of hospitalized COVID-19 patients [144]. In addition, as of 31 August 2020, the efficacy of the investigational Covid 19 vaccine; AZD1222 has been started in 80 sites in United States involving 30000 volunteers [145]. Unusual and admirable efforts of the researchers continue to end the pandemic in the world.

The novel study about the role of corticosteroids in treatment of SARS Cov-2, namely, RECOVERY trial showed that mortality rates of the hospitalized Covid-19 patients who require supplemental oxygen or mechanical ventilation were significantly decreased with early initiation of corticosteroid treatment [146].

### **Conclusion**

Millennium pandemic SARS Cov-2 is globally changed our lives and daily habits of our life. General measures such as patient isolation and supportive care are the mainstay of the SARS Cov-2 treatment. Luckily, encouraging developments are being achieved in terms of discovery of an effective treatment and production of a potent vaccine. We believe it is only a matter of time before we overcome the epidemic in cooperation.

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