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- Abstract: Hortin GL, King C, Kopp J. Quantification of rhesus monkey albumin with assays for human microalbumin [Abstract]. Clin Chem 2000;46:A140-
- Editorial: Demers LM. New biochemical marker for bone disease: is it a breakthrough? [Editorial]. Clin Chem 1992;38:2169–70.
- Letter to the Editor: Davey L, Naidoo L. Urinary screen for acetaminophen (paracetamol) in the presence of N-acetylcysteine [Letter]. Clin Chem 1993;39:2348–9.
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- Book: Personal Author Harrell FE Jr. Regression modeling strategies. New York (NY): Springer; 2001. Bailar JC III, Mosteller F, editors. Medical uses of statistics. 2nd Ed. Boston (MA): NEJM Books; 1992:449 p.
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- Technical Reports Tschantz BA, Moran B. Modeling of the hydrologic transport of mercury in the Upper East Fork Poplar Creek (UEFPC) watershed. Technical Report for Lockheed Martin Energy Systems: Bethesda, MD, September 2004.
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- Epub ahead of print Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.
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Research Article

Laboratory employees' perception of occupational risk factors

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Abstract

Objectives: The purpose of this study was to assess the understanding and views of hospital laboratory workers related to risk factors in their work environment.

Methods: This quantitative analysis included 234 laboratory employees in a university hospital. The data were obtained using a survey consisting of 19 questions: 8 items solicited demographic characteristics of the respondents, 3 questions determined their observations about the work environment, and 8 questions asked about occupational risk perception. Risk perception was evaluated using a scale designed specifically for laboratory employees. Frequency, percentages, and averages were used to present descriptive data. A t-test and analysis of variance were used to analyze occupational risk perception according to participant characteristics and responses.

Results: A total of 162 women and 72 men (mean age: 40-49 years) participated in the study. The most common health problem observed was lower extremity pain. The occupational risk perception level was found to be above average (3.13±0.68). There was a statistically significant difference between the occupational risk perception score and the length of employment in the unit ($p<0.05$); however, no statistical significance was found between occupational risk perception and other variables (gender, age, field of work, education, or length of overall professional experience).

Conclusion: The occupational risk perception score of laboratory workers with 11-16 years of experience was higher than that of more recent employees. Training is known to be effective and would appear to be a valuable investment in the development of risk perception among laboratory employees to ensure a safe and effective environment.

Keywords: Hospital, health workers, laboratory, risk factors

Laboratories can be a source of risk to employee health and safety. Safe working conditions are necessary for healthcare staff to remain healthy and provide good services [1]. Employees in hospitals and healthcare institutions face several sources of risk, including physical, chemical, biological, psychological, and ergonomic factors [2-3]. Physical risk factors include noise, vibration, ventilation, dust, radiation, and improper electrical systems. Laboratory workers may be exposed to toxic, allergic, carcinogenic, or harmful effects of numerous chemicals (such as reagents, disinfectants, drugs, or anesthetics). Blood, tissue, or body fluid samples, as well as medical waste, pose a potential biological risk and represent the most important foci of infection for laboratory workers [4]. An individual's subjective judgment about the characteristics and severity of risks that could threaten their safety is defined as risk perception. A high level of risk perception generally indicates greater practice of safe behaviors [5].

The sensitivity of laboratory workers to work environment risks may differ according to demographic features. The present study examined the occupational risk perception of laboratory workers related to health problems arising from work environment risks and analyzed the effect of demographic characteristics and views on the work environment.

This study was designed to provide laboratory managers with measures to be implemented to mitigate work environment risks and encourage safe behavior.

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Materials and Methods

The survey study was deemed appropriate as the data collection method in the study. After the permission number 65384569/4454 was obtained from the Istanbul University Faculty of Medicine Hospitals where the study was conducted, the questionnaire forms were distributed between 15-26 April 2013 after giving information about the study, and the participants were asked to read and answer these forms.

The quantitative analysis method was used to evaluate the data gathered in this descriptive research study. The survey comprised 2 components: a personal information form and an occupational risk perception scale.

The personal information form consisted of 11 items to record the demographic characteristics of the participants and the features of the work environment.

The occupational risk perception scale used is a subdimension of a risk perception scale developed by the principal author in 2014. The scale consists of 8 items scored using a 5-point Likert scale (1: strongly disagree, 2: disagree, 3: undecided, 4: agree, and 5: strongly agree). The occupational risk perception form used a scoring system of 1-5 for each item, where a score of 5 indicates the highest risk, and the mean was calculated [6-7].

The Cronbach's alpha coefficient for the occupational risk perception scale was 0.73. The significance value of Bartlett's test was χ^2=2249.33 ($p<0.000$). The Kaiser-Mayer-Olkin sampling value of the survey data was determined to be 0.78.

Statistical analysis

The study data were analyzed using SPSS for Windows, Version 15.0 (SPSS Inc., Chicago, IL, USA). Percentages, means, and frequency values were used in evaluation of the meta-data. Independent sample t-test and independent sample analysis of variance were performed in order to analyze the occupational risk perception of the laboratory workers based on their demographic characteristics. Tukey's test was used as post-hoc test to determine any differentiation. Statistical significance was defined as $p<0.05$.

Results

The distribution of the demographic characteristics of the participants is presented in Table 1. In the study group, 40.6% were between the ages of 40 and 49 years, 69.2% were women, 38.5% held an associate degree, and 40.4% were employed as a laboratory technician. The mean length of employment of 44% of the participants had been working in the profession for 17 years. 42.7% of the participants had been employed in the same unit for less than 5 years.

Table 2 illustrates the distribution of responses related to the work environment. It was determined that 65.4% of the participants chose their position (it was not an assignment) and were satisfied.

Table 1. Distribution of the demographic characteristics of the participants (n=234)

Variances	n	Percentage (%)
Female	162	69.2
Male	72	30.8
Age (years)		
20-29	70	29.9
30-39	49	20.9
40-49	95	40.9
≥50	20	8.5
Department		
Emergency laboratory	16	6,8
Biochemistry-genetics research	20	8.5
Microbiology	34	14.5
Blood center	26	11.1
Pediatric biochemistry	19	8.1
Service laboratories	21	9.0
Position		
Biologist-chemist	72	32.0
Other lab worker	15	7.1
Laboratory technician	97	40.4
Nurse	25	9.8
Engineer	6	2.7
Doctor	19	8.1
Education		
Medical vocational high school	30	12.8
Associate's degree	90	38.5
Bachelor's degree	66	28.2
Postgraduate/doctorate and above	48	20.5
Employment in the profession		
0-5 years	61	26.0
6-10 years	35	15.0
11-16 years	35	15.0
≥17 years	103	44.0
Employment in the department		
0-5 years	100	42.7
6-10 years	36	15.4
11-16 years	28	12.0
≥17 years	70	29.9

The participants were asked to identify factors they thought were risks in their work experience: infectious samples, work environment conditions, (noise, lighting, ergonomics, etc.), radiation exposure, chemical use, sharp-object injuries, contact with patients, and inadequate safety protocols/procedures. The most common health problems noted were lower extremity pain, diseases potentially caused by air conditioning quality, and upper extremity pain.

Table 3 provides the distribution of the occupational risk perception. The mean score was 3.13±0.677; the participants perceived their work environment as hazardous. The statement

Table 2. Findings related to the participants' work environment (n=234)

Variances	n	Percentage (%)
Employees' satisfaction level and choice of position		
I chose voluntarily and I am satisfied	153	65.4
I chose voluntarily and I am not satisfied	29	12.4
I did not choose voluntarily but I am satisfied	36	15.4
I did not choose voluntarily and I am not satisfied	16	6.8
Risk factors employees think have an impact on their health in the working environment*		
Infectious samples	133	61.1
Sharp-object injuries	21	9.6
Chemicals	38	17.4
Work environment conditions, (noise, lighting, ergonomics, etc.)	112	52.6
Contact with patient	13	5.9
Inadequate safety protocols/procedure	5	2.3
Health problems experienced by laboratory employees due to the work environment*		
Hepatitis	42	17.9
Eczema	23	9.8
Upper extremity (hand, arm) pain	67	28.6
Varicosis	47	20.1
Psychological disorders	42	17.9
Diseases sourced to air conditioning	76	32.5
Lower extremity (foot, leg) pain	85	36.3
Other	19	8.1

*More than one option could be selected.

Table 3. Distribution of occupational risk perception average (n=234)

Occupational risk perception In the laboratory where I work,	Mean	SD
I think the noise caused by the devices is excessive.	3.59	1.27
I have health problems due to insufficient air conditioning/ventilation.	3.42	1.36
I think that the quality of the personal cleaning agents (hand disinfectants, etc.) used is low, which increases my risk of getting an infection.	3.34	1.32
I think my infection risk is greater because I have contact with patients.	3.27	1.29
I think my infection risk is greater than that of employees of other departments due to exposure to blood and body fluids.	3.13	1.34
I think there are hazardous substances in the workplace that threaten my health.	2.88	1.28
I think I am exposed to infection risk due to working with inadequate equipment and materials.	2.84	1.38
I think I am exposed to radiation (radioactive agents).	2.61	1.45
Totally score	3.13	0.677

"I think the noise caused by the devices is excessive" was the perceived risk with the highest score (3.59±1.27), and the statement "I think I have been exposed to radiation" was the lowest (2.61±1.45).

The distribution of the demographic variance and occupational risk perception is given in Table 4. A comparison of the occupational risk perception scale mean score and demographic characteristics revealed that there was a statistically significant difference only in the duration of employment in the specific department. Participants who had worked in the same department for 11-16 years had a higher risk perception than those employed for 0-5 years ($p<0.05$). There was no significant difference between age, gender, education, marital status, work in another unit, the length of overall employment at the institution, or the duration of employment in the profession.

Discussion

This research of hospital laboratory workers' perceptions of occupational risk factors related to the work environment was performed to provide guidance to anticipate risks that may arise in the future and to take the necessary measures to provide a safe laboratory environment. The results of our survey indicated that 40.6% of the participants were between the ages

Table 4. Comparison of demographic variances and occupational risk perception average (n=234)

	Occupational risk perception (mean±SD)	f	t	p
Gender			1.06	0.5
Female	3.10±0.716			
Male	3.20±0.577			
Age		1.16		0.32
20-29 years	3.15±0.563			
30-39 years	3.28±0.68			
40-49 years	3.05±0.769			
≥50 years	3.13±0.529			
Department	1.072		0.380	
Central laboratory	3.11±0.702			
Emergency laboratory	3.30±0.506			
Biochemistry-genetics research	2.98±0.816			
Microbiology	3.24±0.589			
Blood center	3.27±0.533			
Pediatric biochemistry	3.14±0.600			
Service laboratories	2.92±0.853			
Position		1.493		0.193
Biologist-chemist	3.16±0.579			
Other lab worker	3.08±0.668			
Laboratory technician	3.18±0.712			
Nurse	3.16±0.833			
Engineer	3.29±0.452			
Doctor	2.74±0.626			
Education		0.169		0.918
Medical vocational high school	3.14±0.818			
Associate's degree	3.15±0.710			
Bachelor's degree	3.16±0.584			
Postgraduate –doctorate and above	3.07±0.655			
Employment in the profession		2.369		0. 071
0 - 5 years	3.06±0.552			
6 - 10 years	3.21±0.628			
11 - 16 years	3.39±0.675			
≥17 years	3.07±0.743			
Employment in the department		3.130		0.026*
0 - 5 years(a)	3.04±0.640			
6 - 10 years	3.06±0.721			
11 - 16 years(b)	3.46±0.671			
≥17 years	3.18±0.678			

*P<0.05 was considered significant. Tukey=(b>a). f: Frequency; t: Student's t test.

of 40 and 49 years, 69.2% were women, 38.5% had an associate's degree, and 40.4% were employed as a laboratory technician.

Infection-infected samples, work environment conditions, radiation and chemical exposure, and sharp-object injuries were considered risk factors in the working environment. In other research in Turkey, Çokluk et al. [8] found that the risk factors most cited were infection, sharp-object injuries, contact with body fluids, exposure to chemicals, and musculoskeletal problems. Kılıç et al. [9] reported that the greatest risks in the work environment were sharp-object, biological, psychosocial, physical, and chemical injuries. Pedrosa et al. [10] observed in a study conducted in Brazil that 92% of blood-borne infections occurred in hospitals. According to Gündüz [11], 40.9% of health workers had a work accident; 84.5% reported sharp-object injuries, 33% experienced musculoskeletal injuries, and 36.9% had exposure to contamination with blood or body fluids. Turhan [12] found

that among 988 healthcare employees, 64% were infected at least once due to exposure to blood or body fluid. In our survey, infectious samples were reported as the greatest source of risk. This is consistent with previous studies. However, our study differed from other research in that sharp-object injuries were found to present a low risk.

A study conducted on chemical exposure in a research laboratory in Italy yielded a response that 54.4% felt very exposed to chemical risk [13]. Our results revealed a relatively low ratio of perceived chemical risk (17.4%).

In our research, the most common health problem identified was lower extremity pain (36.3%). Healthcare workers have a significantly greater exposure to musculoskeletal disorders than some other occupations [14]. The number of lower extremity injuries reported in our survey may be related to a lack of sufficient training about how to avoid such injuries. The routine activities of healthcare employees can cause musculoskeletal disorders over the course of time [15].

In our study, noise, insufficient ventilation, contact with dangerous substances, cleaning materials used, and patient contact were perceived as risky. Hazardous materials, inadequate equipment, and radiation were not perceived as great sources of risk. The occupational risk perception scale results indicated that noise was perceived as the greatest risk and radiation exposure was considered the lowest risk. Ilgar [16] also noted that noise ranked first among the important risk factors defined by healthcare professionals. In the study conducted by Vehid et al., [17] noise was a high-risk factor. It has also been reported that medical waste, electrical devices, noise, and air conditioning systems were sources of potential exposure to injury for nurses [18]. In our study, the mean occupational risk perception of nurses was high (3.16±0.833). Results in the literature support our findings.

Aluko et al. [19] reported that 96.2% respondents said they believed they were at risk due to an occupational hazard and 40% stated that the basic safety equipment in the workplace was insufficient. In our study, the laboratory workers perceived the risk due to inadequate equipment to be low.

A comparison of the demographic variables of the participants and the occupational risk perception scores indicated that male employees reported a higher perception of occupational risk than females. Occupational risk perception was found to be higher in the 30-39 age range, those with an associate's degree, those with a title of laboratory technician, and those working in the unit for 11-16 years. Buxton et al. [20] observed that laboratory technicians have significant expertise and experience in the laboratory. However, a study conducted in Egypt in 2019 reported that there was no statistically significant relationship between risk perception score and the frequency of occupational accidents [21].

The occupational risk perception scale used in this study was developed by the principal researcher and to our knowledge, it is the only risk perception scale specifically designed for laboratory workers. The mean score was 3.13±0.677 in our study, which indicates that laboratory workers high risk in the environment.

Saygılı and Çelik [22] used an individual workload perception scale in 2011 and Mollaoğlu et al. [23] assessed the perceptions of nurses working in hospitals about their working environment. A statistically significant relationship was found between the perception of the general work environment and the general level of job satisfaction. According to Taylor and Snyder [24], the relationship between risk perception and safety behavior is uncertain. We found no previous study in the literature specifically examining the occupational risk perception of laboratory workers.

We observed a significant difference between occupational risk perception and the length of time working in the department: The occupational risk perception of the laboratory workers with 11-16 years of experience was higher ($p<0.005$). Considering the role of the training in individuals' behavior, short training sessions at regular intervals may help to develop and maintain greater risk perception. There was no statistically significant difference for the demographic variables of age, gender, department, position, length of employment in the profession, or educational status.

Aktürk and Karadağ [25] stated that there is no relationship between the actual risk faced by the employees and the Employment Period in the department. In our study, a relationship was found between occupational risk perception and length of employment in the unit.

Conclusion

In conclusion, additional, more comprehensive studies should be conducted to eliminate the existing deficiencies regarding the risks faced and perceived by laboratory employees. Training should be provided to inform employees of occupational risks, particularly new laboratory workers, to provide the safest and most effective environment possible.

Conflict of Interest: There is no conflict of interest.

Ethics Committee Approval: This Study permission number 65384569/4454 was obtained from the Istanbul University Cerrahpasa Medical Faculty within the scope of thesis project.

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Research Article

An exploratory study of some pediatric liver function parameters in a segment of North Indian children

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Abstract

Objectives: Western clinical reference levels may not be valid for children in India because of differences in genetic profile, physique, dietary intake, lifestyle, and environmental factors; however, appropriate values for most biochemical parameters for children in India remain unknown. This study is an analysis of the total protein, albumin, globulin, albumin-globulin ratio, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, and alkaline phosphatase (ALP) levels in the serum of a segment of apparently healthy Indian children of aged 0-14 years.

Methods: Liver function data from tests conducted between January and June of 2019 for 371 children were extracted from the laboratory records of a tertiary care hospital and double-filtered to obtain uncontaminated values. The data were statistically tested for significance by age group and sex. Reference ranges of 2.5th and 97.5th percentiles were determined for age groups of 1-4, 5-9, and 10-14 years. Due to the limitation of the sample size, we did not provide a reference range for children aged less than 1 year.

Results: A significant sex difference ($p<0.001$) was observed only for the ALP level. Age group differences were not significant (all $p>0.05$); however, reference ranges were provided for age groups within 1-14 years to provide specificity and assist with comparisons. The liver function values in the study group of Indian children differed from what have been reported for Western children.

Conclusion: The findings are suggestive and not confirmatory, as our sample was insufficient for a determination of definitive values. Nonetheless, the results provide valuable information and indicate a need to carry out more studies to delineate liver function parameters in Indian children since the values may differ from those of other populations.

Keywords: Children, enzymes, liver function, proteins, reference range

Adult medical parameters are rarely applicable to children, due to the smaller body and organ size, typical nutritional requirements, and the process of physiological development. A review of the literature indicated that the biochemical values of Indian children are generally not known. The marked difference in genetic profile, physique, dietary intake, lifestyle, and environmental factors suggests that the values in Indian subjects are likely different from those reported for Western subjects [1]. The preceding statement referred to adults, but applies equally well to children. The results of several studies indicate that some reference values reported for children in different populations reveal wide variation across countries, even when limited to Western countries.

The scope of this study was restricted to some liver function parameters. They reflect different phases of physiological development in children [2] and are used for the diagnosis of conditions such as liver injury, hepatitis, parasitic infection, and Wilson's disease [3], as well as hepatic steatosis [4], including non-alcoholic fatty liver disease. The aim of the present study was to examine the level of various proteins and enzymes in the serum of a segment of apparently healthy children in North India. The objective was to examine how age

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and sex might affect these values in this population of children, whether they are different from those of adults, and whether the values in Indian children differ from counterparts in other countries. Since the liver chemistry of children varies with age and sex and requires customized reference ranges [5], estimates of reference ranges for different groups based on age and sex were provided, though we realize that the current sample is not sufficient in size or a random representative sample of healthy North Indian children. Thus, the reference ranges presented are suggestive and not confirmatory. The authors wish to be fully transparent, as is required for truthful and useful research [6].

In India, children in the general population rarely undergo liver function tests. In addition, ethical considerations restrict researchers from collecting samples from healthy children [7]. The present study used a relatively small sample, and therefore it is merely an exploratory study to generate a hypothesis and to initiate and promote potentially promising directions of further research [8]. It is insufficient to reach any firm conclusion. Since a large-scale study on a nationally representative sample of children is not envisioned in the near future in India, the results of this study could be a unit for a meta-analysis of many such studies conducted in the course of time. Such a meta-analysis can help to reach a firm conclusion regarding liver function parameter values in Indian children.

The liver function tests analyzed were serum levels of total protein, albumin, globulin, albumin-globulin (A/G) ratio, alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP). While the other parameters have well-known uses in the medical evaluation of patients, questions are sometimes raised about the utility of globulin levels and the A/G ratio. Both of these values are calculated and not directly obtained. The globulin levels and the A/G ratio are not as useful as the albumin and enzyme levels, but they provide helpful information when interpreted with levels of total protein. A high globulin level is used to detect paraproteins and low values indicate antibody deficiency [9]. A reduced A/G ratio can indicate inflammation [10].

Materials and Methods

The results of liver function tests performed between January 2019 and June 2019 were retrieved from the laboratory records of a large tertiary care hospital in Delhi-National Capital Region, India. Hospital patients are not an optimal study group, but efforts were made to select values that would ensure the validity of our results. Several studies have used similar hospital data [11-13].

The hospital is a private sector institution and the clientele is generally the more prosperous members of the local community. The hospital is also a member of a group used by several companies to provide preventive health checkups and care for their employees and their families. The test results generally belong to these relatively healthy segments of the population and are appropriate for studying healthy values.

The extracted data set included the liver function test results of more than 18000 individuals. The findings for adults were reported separately [14]. Among these 18000, only 371 were children aged 0-14 years and the present study is based on those test results. Nearly one-third (34.8%) of these patients were female.

The double filtration method [15] was used to assure that uncontaminated, healthy values were analyzed. The technique excludes the values of repeat investigations in the first filtration, and outliers and abnormal values based on box plots are eliminated in the second filtration. The children may have been ill at the time of blood sampling, and were excluded. The second filtration yielded a different number of subjects for various parameters. Many other authors have also excluded abnormal values to determine reference ranges, although different method have been employed for exclusion. For example, Bussler [16] excluded values of more than 3 SD outside the mean for subjects in Germany, and Ridefelt [17] excluded high ALP values to obtain reference values for children in Sweden.

The liver undergoes dramatic changes in structure and function during a child's development [18]; therefore, it is important to use suitable, homogeneous, age intervals. However, there is presently no consensus regarding proper age intervals to reflect the different phases of physiological development [7]. Authors have used different age intervals for different parameters. Li et al. [2] used groups of 2-10 and 11-14 years for albumin level but 2-3, 4-9, 10-12, and 13-14 years for ALP level. In contrast, Gupta [19] reported an albumin level for 1-7 and >7 years, and an ALP level for 2-10 years and adolescents. The Harriet Lane Handbook [20] used age groups of 1-3, 4-6, 7-9, and 10-11 years for ALT level, and 1-2 and 3-16 years for the albumin level. Li et al. [2] used a statistical method to determine homogeneous age-intervals, but the graphs provided in their report do not indicate any significant change in the values according to age intervals. Ridefelt et al. [21] used "qualified guessing" to partition by age and sex. In the absence of a consensus, conventional age intervals, namely, 0-1, 1-4, 5-9 and 10-14 years were used in this study for all of the parameters. This may be more practical, since these are used for many physiological parameters in children.

The age-sex distribution of the initial sample of children is presented in Table 1. The final sample used after double filtration is described in the Results section to avoid duplication.

All of the samples were analyzed on a Cobas 501 autoanalyzer (Roche Diagnostics, Basel, Switzerland) and the standard analytical method was uniformly followed in the laboratory during the entire period of the study to measure levels of the 8 liver function parameters analyzed. Strict internal and external quality control measures were adopted for accurate measurements. Details are provided in our earlier report, which examined adults [14].

Table 1. Age-sex distribution of the initial sample of children

Age group (years)	Male n (%)	Female n (%)	Total n (%)
< 1	38 (15.7)	17 (13.2)	55 (14.8)
1–4	45 (18.6)	35 (27.1)	80 (21.6)
5–9	73 (30.2)	43 (33.3)	116 (31.3)
10–14	86 (35.5)	34 (26.4)	120 (32.3)
Total	242 (100)	129 (100)	371 (100)

Student's t-test was used to evaluate the statistical significance of the difference between the mean in male and female children, but a stricter criterion of p<0.01 (1% level of significance) was applied since there were multiple p values [22]. The age difference in the means was tested using one-way analysis of variance (ANOVA) followed by the Tukey test, also at 1% level of significance.

In place of mean±2 SD [23], the reference range was presented as 2.5th to 97.5th percentiles, which work equally well for both Gaussian and non-Gaussian distributions. This range is also recommended by the Clinical and Laboratory Standard Institute [24]. All of the calculations were performed using IBM SPSS Statistics for Windows, Version 21.0 software (IBM Corp., Armonk, NY, USA).

Ethics approval for this study was granted by the Institutional Ethics Committee of Max Super Specialty Hospital, Vaishali (no: RS/MSSH/VSH/CRL/IEC/PATH/19-19).

Results

The sociodemographic data and the mean and SD of values of different parameters remaining after double filtration are shown in Table 2a and 2b by age group and sex. The p values in the last column of these tables show that the evidence

Table 2a. Mean and SD of total protein, albumin, globulin, and albumin/globulin ratio in children by age and sex

Parameter	Age group (years)	Sex						P value for sex difference
		Male		Female		Total (M+F)		
		n*	Mean (SD)	n*	Mean (SD)	n*	Mean (SD)	
Total protein (g/dL)	<1	22	5.65 (1.25)	15	5.91 (0.83)	37	5.75 (1.09)	0.485
	1-4	37	6.86 (0.61)	31	7.12 (0.49)	68	6.98 (0.57)	0.060
	5-9	48	7.20 (0.63)	27	7.14 (0.58)	75	7.18 (0.61)	0.685
	10-14	57	7.14 (0.71)	29	7.22 (0.67)	86	7.17 (0.69)	0.616
	Combined (0–14)	164	6.90 (0.91)	102	6.98 (0.76)	266	6.93 (0.86)	0.459
P value for age difference			<0.001		<0.001		<0.001	
Albumin (g/dL)	<1	22	3.79 (0.76)	14	4.10 (0.34)	36	3.91 (0.65)	0.161
	1-4	37	4.27 (0.52)	28	4.66 (0.25)	65	4.44 (0.46)	<0.001
	5-9	50	4.27 (0.57)	28	4.21 (0.60)	78	4.25 (0.58)	0.663
	10-14	58	4.38 (0.54)	27	4.32 (0.53)	85	4.36(0.54)	0.633
	Combined (0–14)	167	4.24 (0.60)	97	4.36 (0.50)	264	4.28 (0.57)	0.098
P value for age difference			<0.001		<0.001		<0.001	
Globulin (g/dL)	<1	22	1.86 (0.82)	16	1.87 (0.65)	38	1.86 (0.75)	0.968
	1-4	39	2.59 (0.49)	34	2.54 (0.44)	73	2.57 (0.47)	0.650
	5-9	50	2.80 (0.48)	29	2.88 (0.54)	79	2.83 (0.50)	0.497
	10-14	58	2.73 (0.46)	29	3.00 (0.40)	87	2.82 (0.45)	0.009
	Combined (0–14)	169	2.61 (0.61)	108	2.65 (0.62)	277	2.63 (0.61)	0.597
P value for age difference			<0.001		<0.001		<0.001	
A/G ratio	<1	21	2.21 (0.77)	16	2.27 (0.73)	37	2.23(0.74)	0.812
	1-4	39	1.67 (0.43)	35	1.73 (0.49)	74	1.70(0.46)	0.577
	5-9	50	1.52 (0.31)	29	1.50 (0.40)	79	1.51(0.34)	0.805
	10-14	58	1.64 (0.32)	29	1.44 (0.32)	87	1.57(0.33)	0.007
	Combined (0–14)	168	1.68 (0.47)	109	1.67 (0.54)	277	1.68(0.50)	0.871
P value for age difference			<0.001		<0.001		<0.001	

*After double filtration. A/G: Albumin/globulin; F: Female; M: Male.

Table 2b. Mean and SD of ALT, AST, GGT, and ALP in children by age group and sex

Parameter	Age group (years)	Sex						P value for sex difference
		Male		Female		Total (M+F)		
		n*	Mean (SD)	n*	Mean (SD)	n*	Mean (SD)	
ALT (U/L)	<1	19	21.74 (9.73)	14	17.18 (6.73)	33	19.80 (8.77)	0.142
	1-4	31	18.87 (8.57)	34	18.69 (9.79)	65	18.77 (9.16)	0.938
	5-9	42	20.12 (11.87)	25	21.56 (9.90)	67	20.66 (11.12)	0.612
	10-14	49	22.61 (11.68)	24	18.53 (6.86)	73	21.27 (10.47)	0.119
	Combined (0–14)	141	20.93 (10.87)	97	19.17 (8.78)	238	20.21 (10.09)	0.187
P value for age difference			0.456		0.427		0.513	
AST (U/L)	<1	19	37.91 (13.80)	12	27.58 (8.60)	31	33.91 (12.95)	0.016
	1-4	33	37.71 (12.02)	35	34.97 (11.85)	68	36.30 (11.92)	0.347
	5-9	43	31.40 (11.92)	22	31.80 (6.47)	65	31.54 (10.34)	0.884
	10-14	47	27.34 (10.31)	22	23.14 (4.76)	69	26.00 (9.10)	0.074
	Combined (0–14)	142	32.40 (12.40)	91	30.37 (9.98)	233	31.60 (11.53)	0.191
P value for age difference			<0.001		<0.001		<0.001	
GGT (U/L)	<1	20	76.40 (71.50)	14	65.71 (65.73)	34	72.00 (68.37)	0.661
	1-4	34	13.56 (4.51)	32	11.25 (2.86)	66	12.44 (3.95)	0.016
	5-9	40	14.86 (4.06)	21	15.81 (5.83)	61	15.19 (4.72)	0.488
	10-14	42	15.11 (4.32)	24	17.71 (6.71)	66	16.05 (5.41)	0.060
	Combined (0–14)	136	23.66 (34.91)**	91	22.39 (31.59)**	227	23.15 (33.55)**	0.781
P value for age difference			<0.001		<0.001		<0.001	
ALP (U/L)	<1	21	279.9 (135.4)	15	233.5 (85.3)	36	260.6 (117.9)	0.251
	1-4	36	210.39 (62.16)	34	190.68 (58.82)	70	200.8 (60.9)	0.178
	5-9	43	198.04 (45.32)	28	219.50 (78.30)	71	206.5 (60.9)	0.148
	10-14	57	272.40 (85.17)	26	151.88 (50.67)	83	234.6 (94.3)	<0.001
	Combined (0–14)	157	238.82 (87.51)	103	194.96 (72.33)	260	221.4 (84.5)	<0.001
P value for age difference			<0.001		<0.001		<0.001	

*After double filtration. ** SD is high because of extremely high variation in infants. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; F: Female; GGT: Gamma-glutamyl transferase; M: Male.

for sex difference was not sufficient for any parameter at a 1% level of significance, with the exception of albumin in the age group of 1-4 years, and globulin, A/G ratio, and ALP in the age group of 10-14 years. All other p values for sex difference were >0.01. While a sex difference in the 10-14 group may be due to the differential effects of puberty, the male-female difference in the 1-4 group may be a random error in this study, as it did not persist in other age groups. When all of the age groups were considered together, the p values for age differences were significant ($p<0.01$) for all the parameters in both sexes according to an ANOVA F-test, but the Tukey test pair-wise comparisons at a 1% significance level revealed the following:

(i) On average, the protein levels (total protein, albumin, globulin, and A/G ratio) were significantly different (lower total protein, albumin, and globulin, but higher A/G ratio) in infants, but were not different in the 1-4, 5-9, and 10-14 groups. Thus, a single reference interval may be sufficient for all patients aged 1 to 14 years, but a reference range for each of these age groups was provided for comprehensiveness. The 0-1 group was not defined, as the reliability is not adequate for this age group.

(ii) Among the enzymes, GGT and ALP also exhibited a difference in the mean level in infants in comparison with the other age groups, but no difference was observed between the 1-4, 5-9, and 10-14 groups. The GGT level was considerably higher in infants, and the ALP level was also slightly but significantly higher in infants.

(iii) The mean ALT level was not significantly different in any pairing of age groups. Thus, the same reference range can be used for children aged 0-14 years.

(iv) The AST level was higher on average in the 1-4 age group than in the other age groups, which could be the result of a random error. Thus, the same reference range can be used for all age groups 1-14 years.

The group aged less than 1 year had a large and clinically relevant difference (more than 10%) when compared with the older age groups for most of these parameters. Protein levels, particularly globulin, were lower in both males and females (Table 2a), and GGT levels were considerably higher (more than 4 times) in infants than the older groups (Table 2b). This infant group requires a separate study with an adequately sized sample. Due

to the small sample size in this study, it would be inappropriate to split this age group further into neonates and post-neonates due to potential differences. There are reports in the literature of different values for some of these parameters in young children (see The Harriet Lane Handbook [20] and Gupta [19] for GGTP level and AST level, and Tietz [25] for total protein level). Due to the limitation of the sample size, we did not provide a reference range for children aged less than 1 year.

The reference ranges are provided in Table 3 as 2.5th-97.5th percentile values. The age group of 0-1 was excluded as a result of the small number of children in that group and the consequent poor reliability of reference ranges. The reference ranges were presented without gender differentiation since the difference in means was not statistically significant, with the exception of a lower ALP level in females aged 10-14 years. Ranges were provided for different age groups despite a non-significant difference for comparability with the literature and use in additional studies.

Table 3. Reference range for different liver function parameters in children by age group and sex

	Age (years)	Sex	Reference range
Total protein (g/dL)	1-4	M or F	5.5–8.0
	5-9		5.0–8.4
	10-14		5.7–8.4
Albumin (g/dL)	1-4	M or F	3.2–5.1
	5-9		3.2–5.1
	10-14		3.1–5.2
Globulin (g/dL)	1-4	M or F	1.8–3.6
	5-9		1.9–4.0
	10-14		2.0–3.8
A/G ratio	1-4	M or F	0.65–2.45
	5-9		0.68–2.08
	10-14		0.90–2.18
ALT (U/L)	1-4	M or F	3.9–41.4
	5-9		5.6–47.8
	10-14		8.5–49.3
AST (U/L)	1-4	M or F	15.9–64.1
	5-9		11.8–64.8
	10-14		15.4–62.5
GGT (U/L)	1-4	M or F	
	6.7–25.6		
	5-9		7.6–29.1
	10-14		7.7–31.6
ALP (U/L)	1-4	M or F	81–335
	5-9		96–367
	10-14	M	96–444
		F	77–263

A/G: Albumin-globulin ratio; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; F: Female; GGT: Gamma-glutamyl transferase; M: Male.

The trend of the median and 2.5th and 97.5th percentiles of all 8 parameters in male and female children by age group are shown in Figures 1a and 1b. The age group of 0-1 year was also included to illustrate the difference between values in infants and the other age groups. The figures show that, in general, there was a small increasing trend in the values of total protein, albumin, and particularly globulin in both sexes and a decreasing A/G ratio as age increased from 1-14 years although, as mentioned earlier, the difference across age groups was not statistically significant. Enzymes levels (ALT, AST, GGT and ALP) did not demonstrate any discernible trend from 1-14 years.

Discussion

A literature review did not reveal any study of pediatric values of liver function parameters in India. Studies from around the world include the CALIPER (Canadian Laboratory Initiative on Pediatric Reference Intervals) project [26], the Ridefelt study [27] of data from Sweden, Clifford's research in the USA [28], and Lai et al. in Taiwan [29]. These studies provide various values and none can be considered accurate for Indian children.

Age differentials: Li et al. [2] reported increasing values of total protein and albumin level in Han children (China) as age increased from 2-14 years. Ridefelt et al. [20] also reported a slight increase in albumin level in children of Denmark and Sweden (they did not report total protein level). In contrast, our results with a Tukey test suggested that protein levels (total protein, albumin, and globulin) did not significantly differ between the age groups of 1-4, 5-9, and 10-14 years, but the levels were significantly lower in children younger than 1 year of age. The levels stabilized after the age of 1 year, as also reported by Andropoulos [30] for children in Western countries.

For enzyme levels, Ridefelt et al. [21] reported high ALP and AST levels in infants that dropped after the age of 1 year. The trend in ALP level after 1 year of age is erratic in their report, with unexplained escalations and declines in different pediatric age groups. For AST, they provided a reference range for the age groups of 2-8 and 9-17 years, implying that there are no significant differences within those age-intervals. Li et al. [2] reported a decreasing level of AST as age increased 2-14 years, with increasing GGT values. They also reported an erratic trend in ALP and ALT levels over different pediatric age groups.

We observed steeply high levels of GGT in infants (more than 4 times) and slightly but significantly higher ALP levels in this age group (Table 2b). The results of a Tukey test indicated that the levels of these parameters appeared to stabilize after the age of 1 year, as there were no significant differences in the mean levels in the 1-4, 5-9, or 10-14 groups. The mean ALT levels were not significantly different in any age group 0-14 years. The mean AST level was significantly higher in the 1-4 age group, but that would seem to be a random error because there is no known biological reason for this result in this particular age group and it did not persist in other age groups.

Table 4a. Reference interval for total protein, albumin, and globulin reported in the literature and comparison with our values

Parameter	Ref	Age	Sex	Ref range	Ref	Age	Sex	Ref range	Ref	Age	Sex	Ref range	Ref	Age	Sex	Ref range
Total protein (g/dL)	Our study	1-4y	F+M	5.5-8.0	Harriet Lane Handbook	0-15d	F+M	4.4-7.6	Gupta	Term neonate	F+M	4.6-7.7	Burtis and Bruns	Newborn	F+M	4.6-7.0
		5-9y	F+M	5.0-8.4		15d-1y	F+M	5.1-7.3		1-7y	F+M	6.1-7.9		1w	F+M	4.4-7.6
		10-14y	F+M	5.7-8.4		1-2y	F+M	5.6-7.5		>7y	F+M	6.4-8.2		7m-1y	F+M	5.1-7.3
						3-16y	F+M	6.0-8.0						1-2y	F+M	5.6–7.5
														>2y	F+M	6.0–8.0
Albumin (g/dL)		1-4y	F+M	3.2-5.1		0-15d	F+M	3.0-3.9		Term neonate	F+M	2.5-5.0		0-4d	F+M	2.8-4.4
		5-9y	F+M	3.2-5.1		15d-1y	F+M	2.2-4.8		1-7y	F+M	4.0-5.0		4d-14y	F+M	3.8-5.4
		10-14y	F+M	3.1-5.2		1-2y	F+M	3.6-5.2		>7y	F+M	3.4-5.0				
						3-16y	F+M	3.6-5.2								

d: Days; m: Months; Ref: Reference; w: Weeks; y: Years.

Table 4b. Reference interval for ALT, AST, GGT, and ALP reported in the literature and comparison with our values

Parameter	Ref	Age	Sex	Ref range	Ref	Age	Sex	Ref range	Ref	Age	Sex	Ref range	Ref	Age	Sex	Ref range
ALT (U/L)	Our study	1-4y	F+M	3.9-41.4	Harriet Lane Hand-book	<12m	F+M	13-45	Gupta	0-7d	F+M	25-100	Burtis and Bruns	Not given for children		
		5-9y	F+M	5.6-47.8		1-3y	F+M	5-45		8-30d	F+M	22-71				
		10-14y	F+M	8.5-49.3		4-6y	F+M	10-25		1-3y	F+M	20-60				
						7-9y	F+M	10-35		3-9y	F+M	15-50				
						10-11y	F	10-30		10-15y	F+M	10-40				
							M	10-35								
						12-13y	F	10-30								
							M	10-55								
						13-14y	Not given									
AST (U/L)		1-4y	F+M	15.9-64.1		0-10d	F+M	47-150		0-7d	F+M	25-100		Not given for children		
		5-9y	F+M	11.8-64.8		10d-24m	F+M	9-80		8-30d	F+M	22-71				
		10-14y	F+M	15.4-62.5		>24m	F	13-35		1-3y	F+M	20-60				
							M	15-40		3-9y	F+M	15-50				
						10-15y	F+M	10-40								
GGT (U/L)		1-4y	F+M	6.7-25.6		0-1m	F+M	13-147		<3w	F+M	0-130		Not given for children		
		5-9y	F+M	7.6-29.1		1-2m	F+M	12-123		3w-3m	F+M	4-120				
		10-14y	F+M	7.7-31.6		2-4m	F+M	8-90		>3 m	F	5-35				
						4m-10y	F+M	5-32			M	5-65				
						10-15y	F+M	5-24		1-15y	F+M	0-23				
ALP (U/L)		1-4y	F+M	81-335		<12m	F+M	150-420		Term neonate	F+M	<700		0-3y	Not given	
		5-9y	F+M	96-367		1y+	Not given			Infant	F+M	150-420		4-15y	F	54-369
		10-14y	M	96-444						2-10y	F+M	100-320			M	54-369
			F	77-263						10-15y	F	100-320				
											M	100-390				

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; d: Days; F: Female; GGT: Gamma-glutamyl transferase; M: Male; m: Months; Ref: Reference; w: Weeks; y: Years.

Sex differentials: There was no statistically significant difference between the male and female children in our study at the strict significance level of 1%, with the exception of a lower mean ALP level in the females of the 10-14 age group (Table 2b). Ridefelt [21] reported a common reference range for albumin, ALT, ALP, and AST for male and female children up to age 14 in Denmark and Sweden, suggesting that there is no significant difference between the levels in male and female children in that population. The Harriet Lane Handbook [20] uses the same levels of ALT, ALP, AST, GGT, total protein, and albumin for male and female children. Thus, our finding of significantly different levels of ALP in boys and girls aged 10-14 years needs further investigation. Akirov [31] also observed different peak ALP levels in boys and girls of this age.

Comparison with other countries: Table 4a and 4b provide the reference range reported by different authors and the values found in our study. Globulin and A/G ratio values were excluded from these tables, as not much information was available.

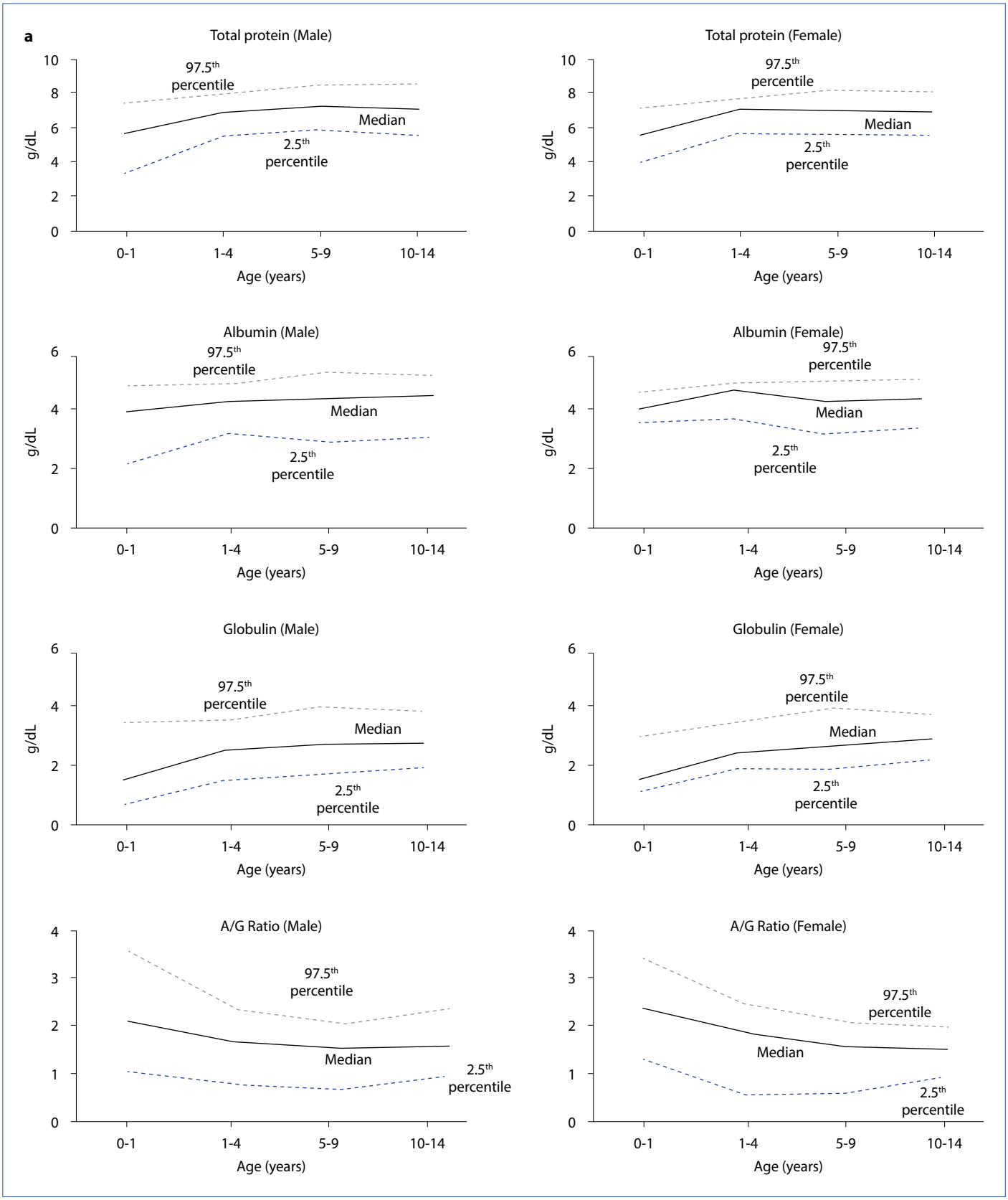

Figure 1a. Age-sex trend of the median and reference range for the levels of total protein, albumin, globulin, and the albumin-globulin ratio in children.

A/G: Albumin-globulin.

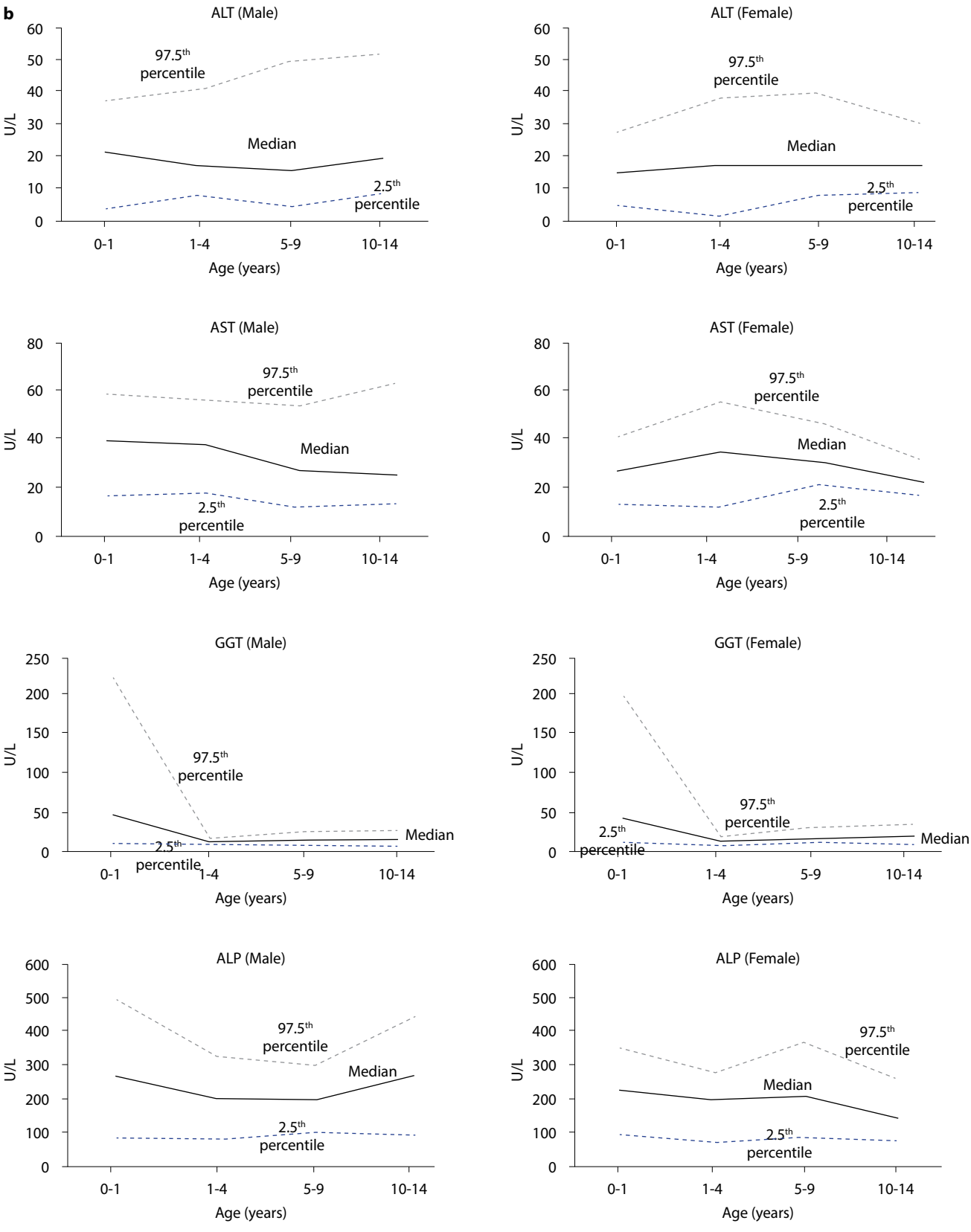

Figure 1b. Age-sex trend of the median and reference range for the levels of ALT, AST, GGT, and ALP in children.

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase.

The wide variation across different reports suggests that values for children need to be studied with more precision, and that age and sex need to be clearly specified where differences exist. At present there is no clarity. The reference ranges obtained in our study are also different. Total protein and albumin levels were slightly lower in our study group than other children of corresponding age, but other parameters did not follow any consistent pattern and the values were different. More such studies are needed to establish precise reference ranges for Indian children.

Comparison of the levels in children with values previously reported for adults of the same population have revealed that generally, the levels of proteins (total protein, albumin, globulin, and A/G ratio) were nearly the same in children aged 1-14 years as in adults, but there was some variation with regard to enzymes levels [14]. The AST and GGT levels were slightly lower, and the levels of AST and ALP were slightly higher in children than in adults. There is no comparable study for India, and there may also be regional differences. If this difference is substantiated, the biological reason should be investigated.

The results for children in other countries are not uniform. We have cited some publications that are generally consulted for reference ranges. The Harriet Lane Handbook [24] reported lower values of total protein, albumin, and GGT in children aged 0-14 years compared with adults, but very similar ALT and AST levels. It also reports slightly higher levels of ALP in children than adults. Tietz [25] reported slightly lower values of total protein in children, but not in other parameters. Our results for a segment of Indian children are different and do not conform to any of the values previously reported. This could be a result of sampling error, but the previously published values also differ from one another. Such variations in the literature from different settings further underscores the need to carry out a large-scale study with a representative sample of children in India to resolve this issue.

Conclusion

To the best of our knowledge, this study is the first in the literature to investigate liver function parameters in Indian children. We analyzed the levels of total protein, albumin, globulin, A/G ratio, AST, ALT, ALP, and GGT in apparently healthy children aged 0-14 years. Though we used a sample of more than 300, we are calling it an exploratory study since science requires modesty [32]. The study has generated interesting hypotheses for further investigation. First, the GGT level in infants was nearly 4 times the level in children 1-14 years of age. Second, there was no significant male-female difference in any of the 8 liver function parameters examined in this study, except the ALP level in the group aged 10-14 years. Third, the protein levels (total protein, albumin, globulin, and A/G ratio) were nearly the same in children of 1-14 years as in adults, but the AST and GGT levels were slightly lower in children, and the AST and ALP levels were slightly higher. Lastly, we provide preliminary reference ranges for these parameters for Indian children aged 1-4, 5-9 and 10-14 years. These are different from previously reported values. Due to the limitation of a relatively small sample, our reference ranges are suggestive and not confirmatory. Further studies will confirm or deny our findings and add to the comprehensive knowledge.

Conflict of Interest: The authors declare no conflict of interest.

Ethics Committee Approval: This study was approved by the Institutional Ethics Committee of Max Super Specialty Hospital, Vaishali (no: RS/MSSH/VSH/CRL/IEC/PATH/19-19).

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Research Article

Laboratory findings in predicting intensive care need and death of COVID-19 patients

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Abstract

Objectives: The ability to predict the course of COVID-19 is very valuable in terms of the optimal use of health resources. The aim of this study was to examine the value of biochemical and hematological parameters in the estimation of hospital stay, disease severity, and likelihood of death.

Methods: Routine blood analysis data of confirmed COVID-19 cases (n=222) were collected and analyzed. The patients were divided into 3 groups: outpatient, inpatient, and patients requiring intensive care.

Results: There were significant differences between the 3 groups in terms of age, lymphocyte, neutrophil, hemoglobin, hematocrit, mean corpuscular volume (MCV), red blood cell distribution width (RDW), neutrophil-to-lymphocyte ratio (NLR), neutrophil-to-monocyte ratio (NMR), platelet-to-lymphocyte ratio (PLR), procalcitonin, C-reactive protein (CRP), and D-dimer values. Univariate analysis for mortality revealed significant differences in neutrophil, NLR, PLR, NMR, procalcitonin, and CRP values. Multivariable logistic regression yielded significant differences in only NMR and procalcitonin values. A positive correlation was determined between the length of hospital stay and age, MPV, procalcitonin, and D-dimer values.

Conclusion: The neutrophil count was the most appropriate parameter to predict the need for intensive care (area under the curve: 0.782, sensitivity: 73%, specificity: 75%, with a cutoff of 4.43). The NMR and procalcitonin values were significant to predict death in multivariate analysis. Age, CRP, and D-dimer values were the parameters most associated with the duration of hospitalization.

Keywords: COVID-19, death, hemogram, intensive care unit, neutrophil

The Coronavirus 2019 (COVID-19) outbreak began in December 2019 in Wuhan, China. Despite efforts to contain it, the epidemic spread around the world. On March 11, 2020, the World Health Organization (WHO) confirmed a pandemic. The number of coronavirus cases had reached 65 million and the number of deaths attributed to the disease was 1.5 million worldwide in December 2020 [1].

Coronaviruses are an infectious agent for the common cold with subgroups that differ in contagiousness and risk of death. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes the illness coronavirus 2019 (COVID-19), is 10-20 times more transmissible than the original SARS-CoV [2, 3]. Countries across the globe are struggling to cope with economic difficulties caused by quarantine measures, as well as health resource constraints, such as insufficient medical facilities and healthcare personnel. Clinical and laboratory findings that can provide a reliable COVID-19 prognosis will help to perform risk stratification to distinguish patients at high risk of developing serious disease. It will also provide guidance for the best possible management of health resources [4]. The identification of laboratory parameters that can be used to predict the severity or

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the mortality risk will provide enhanced clinical situational awareness and facilitate appropriate treatment planning. Greater ability to manage care will reduce the disruption in the health system [5].

Abnormal hematology and biochemistry parameters can be used to diagnose infection-related tissue and organ damage and categorize patients at greater risk of developing severe disease [6]. They can also be used to recognize patients with a high probability of a poor prognosis and in monitoring the course of the disease. Studies have found that several parameters, such as white blood cell count, lymphocyte count, platelet count, and levels of interleukin-6, serum ferritin, and procalcitonin, can help predict the severity of COVID-19 [7, 8].

The objective of this study was to examine the use of biochemical and hematological parameter data of hospitalization, disease severity, and mortality to predict disease progression in 222 patients with a positive polymerase chain reaction (PCR) test result for SARS-CoV-2.

The Turkish Ministry of Health COVID-19 adult patient treatment guideline specifies the criteria for hospitalization criteria for admission to the intensive care unit and intensive care treatment: dyspnea and respiratory distress, respiratory rate ≥30/minute, partial pressure of arterial oxygen (PaO_2) to fraction of inspired oxygen ratio <300 mmHg, increased need for oxygen during monitoring, oxygen saturation (SpO_2) <90% or PaO_2 <70 mmHg despite 5 L/minute oxygen therapy, hypotension (systolic blood pressure [SBP] <90 mmHg, drop of >40 mmHg from normal SBP, and mean arterial pressure <65 mmHg), tachycardia >100/minute, acute kidney damage, acute liver function test disorder, signs of acute organ dysfunction such as confusion, acute bleeding diathesis, and patients with immunosuppression, troponin elevation and arrhythmia, lactate >2 mmol, or the presence of skin disorders such as cutis marmaratus. The decision to stay in the ICU is made by the intensive care medical officer. The criteria for hospitalization are mild-moderate pneumonia with a respiratory rate ≥24/minute and SpO_2 ≤93%, mild-moderate pneumonia and blood values showing poor prognosis (blood lymphocyte count <800/µl, serum C-reactive protein [CRP] >10x normal upper limit, ferritin >500ng/mL, D-dimer >1000 ng/mL, etc.), severe pneumonia (change in consciousness, respiratory distress, respiratory rate ≥30/minute, SpO_2 <90% in room air, lung imaging of bilateral diffuse [>50%] involvement), hypotension (<90/60 mmHg, mean blood pressure <65 mmHg), tachycardia (>100/minute), sepsis, septic shock, myocarditis, acute coronary syndrome, arrhythmia, or acute kidney damage [9].

Materials and Methods

This study was approved by the Health Sciences University Sisli Hamidiye Etfal Training and Research Hospital Clinical Research Ethics Committee on September 22, 2020 (no: 3002). The need for written, informed consent was waived by the hospital ethics committee due to pandemic.

Patients and data collection

A total of 222 patients who were admitted to Sisli Hamidiye Etfal Training and Research Hospital between March 15, 2020 and June 15, 2020 with a positive COVID-19 PCR test and were not pregnant were included in the study. Routine biochemical and complete blood count parameters of COVID-19 patients were studied using a Beckman Coulter AU680 chemistry analyzer (Beckman Coulter, Inc., Brea, CA, USA) and a Mindray BC 6800 hematology analyzer (Mindray Medical International Co. Ltd., Shenzhen, China). Patient information and laboratory results were obtained retrospectively from the hospital and laboratory information management systems. The enrolled patients were divided into 3 groups: outpatients, inpatients, or patients needing intensive care. The hematological and biochemical data of the patients who received outpatient or inpatient treatment were recorded at the time of admission to the hospital. Data recorded on the first day in the ICU were used for patients who received intensive care.

Statistical analysis

The Shapiro-Wilk test was applied to determine the normality of distribution. The results were presented as mean±SD or median (minimum-maximum) for continuous variables. Categorical variables were described as frequency and percentage. Normally distributed data were compared with an independent samples t-test or one-way analysis of variance. The Kruskal-Wallis and Mann-Whitney U tests were used for nonnormally distributed data. The Bonferroni test was used for multiple comparisons. Categorical variables were compared between groups using Pearson's chi-squared test and Fisher's exact test. Correlations between variables were tested using the Spearman correlation coefficient. Univariate logistic regression analysis was performed to assess the association between variables and disease progression. The odds ratio (OR) confidence intervals (CIs) were calculated at 95%. Multivariate backward stepwise logistic regression analysis provided a second estimate of the OR after adjustment for confounding variables. Receiver operating characteristic (ROC) curve analysis of the statistically significant variables after binary logistic regression analysis was performed, and the area under curve (AUC) was calculated to evaluate the sensitivity and specificity of each variable/model to predict the severity of COVID-19. Cox proportional hazards regression model (forward step likelihood ratio approach) was used to perform multifactor analysis and calculate the hazard ratio (HR) values and 95% CIs of the risk factors that were chosen on the basis of likely and relevant confounders after univariate analysis. The level of statistical significance was α=0.05. The statistical analyses were performed with IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA).

Results

The median age of the patients was 56 years (range: 15-97 years) and 49.5% were men. Of the 222 patients, 138 had no

Table 1. Demographic data of all of the study patients

	All patients (n=222)	Outpatient (n=74)	Inpatient (n=111)	ICU (n=37)	p
Age (years)	56 (15-97)	38.5 (15-87)[a]	60 (25-97)[b]	62 (46-93)[b]	<0.001
Gender (female)	110 (49.5%)	42 (56.8%)	56 (50.5%)	12 (32%)	0.052
Any comorbidity	84 (%37.84)	0 (%0)[a]	61 (%54.95)[b]	23 (%62.16)[b]	<0.001
Diabetes	29 (%13.06)	0 (%0)[a]	24 (%21.62)[b]	5 (%13.51)[b]	<0.001
Hypertension	52 (%23.42)	0 (%0)[a]	39 (%35.14)[b]	13 (%35.14)[b]	<0.001
Cardiovascular disease	7 (%3.15)	0 (%0)[a]	3 (%2.70)[ab]	4 (%10.81)[b]	0.012
Chronic obstructive pulmonary disease	9 (%4.05)	0 (%0)[a]	6 (%5.41)[ab]	3 (%8.11)[b]	0.043
Malignancy	2 (%0.90)	0 (%0)	2 (%1.80)	0 (%0)	-
Chronic liver disease	1 (%0.45)	0 (%0)	1 (%0.90)	0 (%0)	-
Exitus	19 (%8.56)	0 (%0)[a]	0 (%0)[a]	19 (%51.35)[b]	<0.001

Descriptive statistics are presented as median (minimum-maximum) or frequency with percentage. ICU: Intensive care unit.

comorbidities; 54% of the inpatients and 62% of the ICU patients had additional comorbid diseases. Diabetes mellitus (23%) and hypertension (13%) were the most common comorbidities. There were significant differences between the groups in age and the rates of comorbidities, diabetes, hypertension, cardiovascular disease, and chronic obstructive pulmonary disease. A total of 26 patients were transferred from inpatient clinics to the ICU and 11 patients were treated in the ICU upon presentation. In all, 51% of the ICU patients died while they were in hospital. There was a significant difference in the mortality rate between groups. The demographic data of the study group is shown in Table 1.

Biochemical and hematological data from the time of admission to the hospital were analyzed (Table 2). CRP, D-dimer, and procalcitonin parameters that were thought to be related to the clinical course were also analyzed to determine any association with hematological parameters. Significant differences between the 3 groups (outpatient, inpatient, ICU) were observed in age as well as lymphocyte, neutrophil, hemoglobin, hematocrit, mean corpuscular volume (MCV), red blood cell distribution width (RDW), neutrophil-to-lymphocyte ratio (NLR), neutrophil-to-monocyte ratio (NMR), platelet-to-lymphocyte ratio (PLR), procalcitonin, CRP, and D-dimer values. Pairwise comparisons revealed that there were significant differences in the NLR, procalcitonin, and D-dimer values of the 3 groups.

The relationship between parameters and admission to the ICU was analyzed. There was a significant difference in terms of age (p=0.001) or gender (p=0.025) between those with and without the need for intensive care. Data adjusted for age and gender were also evaluated using univariate analysis; the hemoglobin and hematocrit levels were low, while the neutrophil, mean platelet volume (MPV), platelet distribution width (PDW), MCV, NLR, PLR, NMR, procalcitonin, CRP, and D-dimer values were high. Multivariate analysis of intensive care need indicated a significant difference in age, MPV, and NMR values (Table 3).

ROC analysis of the parameters was performed to predict the need for intensive care treatment (Table 4). The parameter with the highest AUC was the neutrophil count, with a 73% sensitivity and a 75% specificity for a cutoff level of 4.43 (Fig. 1). The AUC was 0.77 for NLR (sensitivity: 71%, specificity: 79%) (Fig. 2) and the AUC was 0.678 for the NMR of 17.6 (sensitivity: 43%, specificity: 89%) (Fig. 3). In a logistic regression model of MPV and NMR, the AUC was lower than that of the neutrophil count.

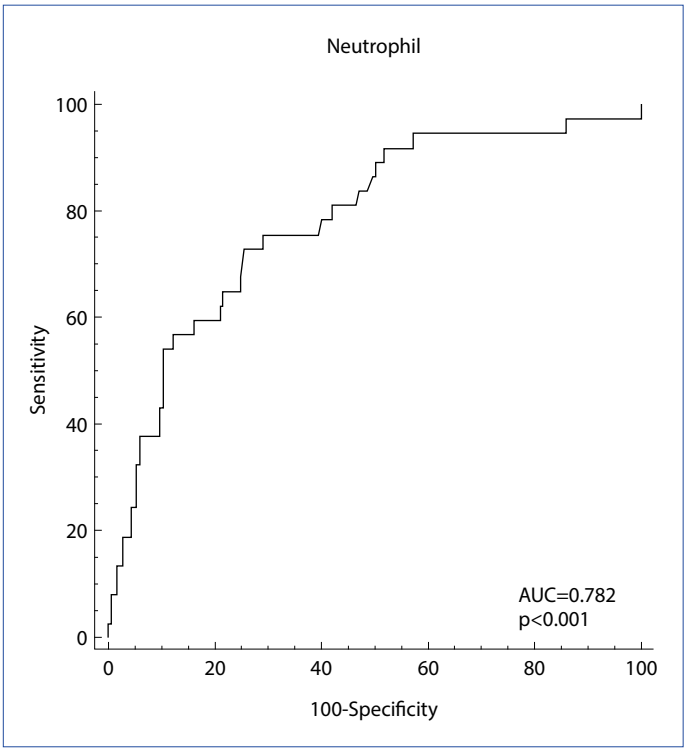

Figure 1. Receiver operating characteristic curve analysis for the neutrophil count as a predictor of the need for intensive care unit hospitalization.

AUC: Area under the curve.

Table 2. Biochemical and hematological data of the patients

	Outpatient (n=74)	Inpatient (n=111)	ICU (n=37)	Total (n=222)	p
Lymphocyte (×10³/µL)	1.56 (0.51-4.25)[a]	1.25 (0.35-8)[a]	1.09 (0.27-4.4)[b]	1.3(0.27-8)	<0.001
Neutrophil (×10³/µL)	3.27 (1.35-6.93)[a]	3.71 (0.89-23.43)[a]	6.41 (0.72-34.48)[b]	3.71 (0.72-34.48)	<0.001
Platelet (×10³/µL)	198 (80-354)	179 (49-552)	206 (70-472)	191 (49-552)	0.227
RDW (%)	12.9 (11.3-17.4)[a]	13.7 (12-22.5)[b]	13.7 (12.3-18.1)[b]	13.5 (11.3-22.5)	0.001
Monocyte (×10³/µL)	0.42 (0.14-0.92)	0.37 (0.06-1.15)	0.39 (0.11-1.46)	0.39 (0.06-1.46)	0.128
Hemoglobin (g/L)	137 (105-173)[a]	130 (74-168)[b]	116 (73-164)[b]	132 (73-173)	<0.001
Hematocrit (%)	41 (32.8-50.3)[a]	39.4 (23.9-51.3)[b]	36.5 (24.2-86.3)[b]	39.55 (23.9-86.3)	0.001
MPV (fL)	9.3 (7.7-12.4)	9.4 (7.2-12)	9.6 (7.4-12.2)	9.4 (7.2-12.4)	0.091
PDW (%)	16.2 (14.8-17.1)	16.1 (15.3-19.1)	16.3 (15.6-17.6)	16.2 (14.8-19.1)	0.069
MCV (fL)	88.1 (57.8-105.6)[a]	87 (59-101.6)[ab]	89.7 (78.5-100.8)[ac]	87.8 (57.8-105.6)	0.026
NLR	2.01 (1-9.1)[a]	3.12 (0.55-66.94)[b]	5.34 (1.48-31.56)[c]	2.80 (0.55-66.94)	<0.001
PLR	130 (66.5-365.8)[a]	152.3 (28.7-771.1)[b]	167.8 (31.6-2568.1)[b]	141.35 (28.7-2568.1)	0.001
NMR	7.55 (2.6-30.3)[a]	10.1 (2.9-57.1)[b]	12.8 (4.7-84.7)[b]	9.3 (2.6-84.7)	<0.001
Procalcitonin (ng/mL)	0 (0-1.8)[a]	0 (0-0.87)[b]	0.15 (0-14)[c]	0 (0-14)	<0.001
CRP (mg/L)	6.1 (0.3-184.4)[a]	30.7 (1.3-256.3)[b]	74 (1.5-374)[b]	18.64 (0.3-374)	<0.001
D-dimer (µg/L)	315.5 (97-1880)[a]	683.5 (67-17700)[b]	1280 (123-7960)[c]	549 (67-17700)	<0.001

Descriptive statistics are presented as median (minimum-maximum). Pairwise comparisons are shown with "a", "b", "c" symbols. CRP: C-reactive protein; ICU: Intensive care unit; MCV: Mean corpuscular volume; MPV: Mean platelet volume; NLR: Neutrophil-to-lymphocyte ratio; NMR: Neutrophil-to-monocyte ratio; PDW: Platelet distribution width; PLR: Platelet-to-lymphocyte ratio; RDW: Red blood cell distribution width.

Table 3. Univariate and multivariate analysis of patient need for intensive care unit hospitalization

Variables	Univariate		Multivariate	
	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
Age	1.04 (1.02-1.06)	0.001	1.02 (1.00-1.05)	0.053
Gender (female)	2.35 (1.113-4.95)	0.025	-	-
Lymphocyte	0.73 (0.41-1.30)	0.286	-	-
Neutrophil	1.28 (1.14-1.45)	0.001		
Platelet	1.00 (0.99-1.00)	0.108	-	-
RDW	1.16 (0.96- 1.41)	0.126	-	-
Monocyte	3.58 (0.70-18.36)	0.126	-	-
Hemoglobin	0.97 (0.95-0.99)	0.001		
Hematocrit	0.94 (0.88-0.99)	0.038	-	-
MPV	1.45 (1.04-2.03)	0.030	1.69 (1.07-2.69)	0.023
PDW	2.32 (1.09-4.94)	0.029	-	-
MCV	1.08 (1.015-1.15)	0.015	-	-
NLR	1.09 (1.02-1.16)	0.009	-	-
PLR	1.003 (1.001-1.006)	0.012	-	-
NMR	1.07 (1.0-1.10)	<0.001	1.07 (1.03-1.12)	<0.001
Procalcitonin	7.42 (1.68-32.74)	0.008	-	-
CRP	1.012 (1.01-1.02)	0.003	-	-
D-dimer	1.00 (1.00-1.00)	0.020	-	-

CRP: C-reactive protein; MCV: Mean corpuscular volume; MPV: Mean platelet volume; NLR: Neutrophil-to-lymphocyte ratio; NMR: Neutrophil-to-monocyte ratio; PDW: Platelet distribution width; PLR: Platelet-to-lymphocyte ratio; RDW: Red blood cell distribution width.

The patients were followed up throughout their treatment and approximately half of patients in ICU died. Parameters to predict death were evaluated using univariate and multivariate analysis. There were significant differences in the neutrophil, NLR, PLR, NMR, procalcitonin, and CRP levels in univariate analysis with values adjusted for age and sex. Multivariable

Table 4. Receiver operating characteristic curve analysis of parameters to predict the need for intensive care treatment

Variables	AUC	p	Optimal threshold	Sensitivity	Specifity	Youden
Age	0.695	<0.001	>55	83.33	54.05	0.3739
Neutrophil	0.782	<0.001	>4.43	73	74.6	0.4757
Hemoglobin	0.654	0.005	≤112	48.65	85.95	0.3459
Hematocrit	0.639	0.016	≤34.8	45.9	86.5	0.3243
PDW	0.618	0.020	>16.1	72.97	49.73	0.2270
MCV	0.639	0.004	>88.5	70.3	58.4	0.2865
NLR	0.772	<0.001	>4.26	71.4	79.5	0.5089
PLR	0.623	0.027	>145.9	70.3	56.8	0.2703
NMR	0.678	0.001	>17.6	43.24	89.19	0.3243
Procalcitonin	0.687	0.001	>0	59.38	71.84	0.3121
CRP	0.727	<0.001	>51.9	62.16	75.41	0.3757
D-dimer	0.746	<0.001	>503	87.88	5.41	0.4029
Logistic regression model	0.766	<0.001	0.137	72.22	74.05	0.4628

AUC: Area under the curve; CRP: C-reactive protein; MCV: Mean corpuscular volume; NLR: Neutrophil-to-lymphocyte ratio; NMR: Neutrophil-to-monocyte ratio; PDW: Platelet distribution width; PLR: Platelet-to-lymphocyte ratio.

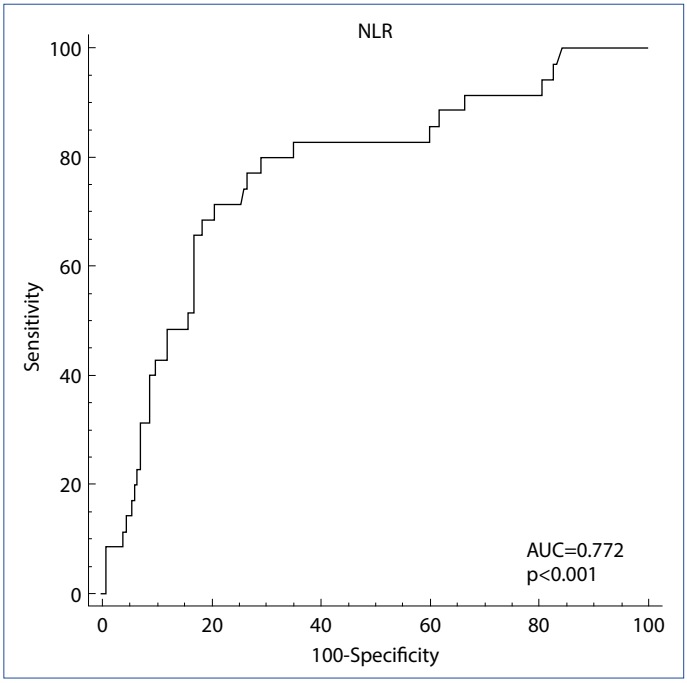

Figure 2. Receiver operating characteristic curve analysis for the neutrophil-to-lymphocyte ratio as a predictor of the need for intensive care unit hospitalization.

AUC: Area under the curve; NLR: Neutrophil-to-lymphocyte ratio.

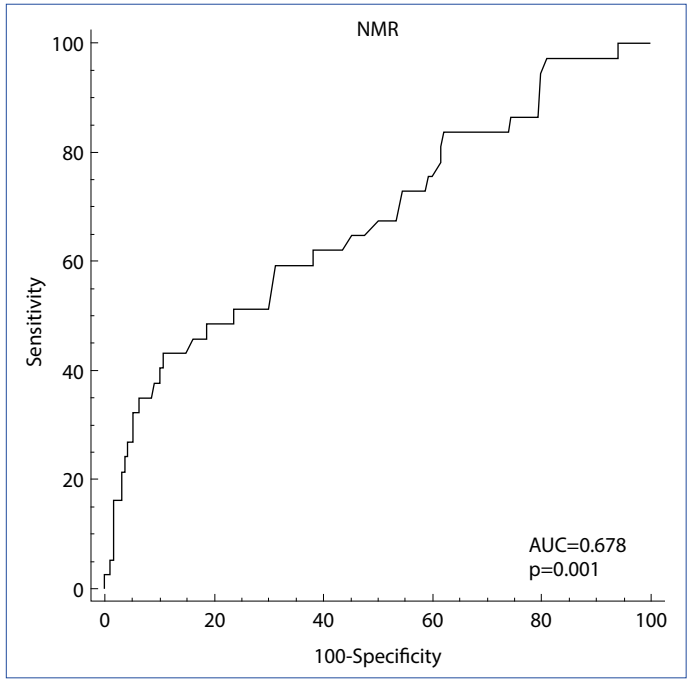

Figure 3. Receiver operating characteristic curve analysis for the neutrophil-to-monocyte ratio as a predictor of the need for intensive care unit hospitalization.

AUC: Area under the curve; NMR: Neutrophil-to-monocyte ratio.

logistic regression revealed a significant difference in only the NMR (HR: 1.05, 95% CI: 1.03-1.08) and procalcitonin (HR: 1.23, 95% CI: 1.03-1.48) values (Table 5).

When the effect of parameters on hospitalization time was examined, it was noted that age, MPV, procalcitonin, CRP, and D-dimer levels (r=0.429, 0.191, 0.192, 0,259, 0,368, respectively, p<0.05) were positively correlated with the length of hospital stay.

Discussion

The ability to develop a reliable prognosis at the time of admission will prevent unnecessary hospitalization and help to ensure the optimal use of resources. An early diagnosis and determination of a prognosis is of critical importance in COVID-19 cases. Many studies have been conducted examining the severity of COVID-19; this research was designed to

Table 5. Univariate and multivariate analysis to predict mortality

Variables	Univariate		Multivariate	
	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p
Age	1.02 (0.989-1.043)	0.251	-	-
Gender (female)	2.48 (0.89-6.87)	0.082	-	-
Lymphocyte	0.40 (0.15-1.05)	0.063	-	-
Neutrophil	1.11 (1.05-1.18)	0.001	-	-
Platelet	1.00 (0.99-1.01)	0.546	-	-
RDW	0.95 (0.72-1.25)	0.706	-	-
Monocyte	0.39 (0.03-5.14)	0.476	-	-
Hemoglobin	0.98 (0.96-1.00)	0.066	-	-
MPV	1.49 (0.98-2.25)	0.059		
PDW	1.74 (0.88-3.43)	0.108	-	-
MCV	1.02 (0.96-1.09)	0.528	-	-
NLR	1.04 (1.01-1.06)	0.022	-	-
PLR	1.001 (1.000-1.002)	0.006	-	-
NMR	1.06 (1.03-1.08)	<0.001	1.05 (1.03-1.08)	<0.001
Procalcitonin	1.42 (1.23-1.63)	<0.001	1.23 (1.03-1.48)	0.025
CRP	1.01 (1.005-1.015)	<0.001	-	-
D-dimer	1.00 (1.00-1.00)	0.237	-	-

CRP: C-reactive protein; MCV: Mean corpuscular volume; MPV: Mean platelet volume; NLR: Neutrophil-to-lymphocyte ratio; NMR: Neutrophil-to-monocyte ratio; PDW: Platelet distribution width; PLR: Platelet-to-lymphocyte ratio; RDW: Red blood cell distribution width.

help predict both the need for intensive care treatment and patient mortality.

In this study, although the lymphocyte count was significantly lower in the patients who required ICU care and the inpatients (p=0.003 and p<0.001, respectively), it was not sufficient to predict the need for intensive care or death. Reports in the literature have indicated that a low lymphocyte count, especially a low T lymphocyte count, was very common in ICU patients. There are varied opinions about this change in lymphocyte count, including that it may be a result of an inflammatory cytokine storm, and that SARS-CoV-2 infection may interfere with T cells, causing depletion, cell infection, or reduced expansion [10]. Wagner et al. [11] noted that a low lymphocyte percentage was a prognostic marker in COVID-19 patients. Several studies have observed that severe cases presented with a low lymphocyte count, a high neutrophil count, and a high NLR [12]. Liu et al. [13] found in a study of 61 patients that the severity of COVID-19 was associated with the NLR and a cutoff value of 3.13 served to indicate severity. Ciccullo et al. [14] reported that NLR was a useful prognostic factor in the early screening of critical illness. Our findings were consistent with the results of previous studies; the NLR was significantly greater in the ICU patients than the inpatients or outpatients (p<0.001). The NLR was also greater in inpatients than outpatients. The NLR was a significant parameter in predicting the need for intensive care and death. ROC analysis with a cutoff 4.26 indicated that NLR was a valuable predictor of the need for ICU hospitalization (AUC: 0.77, sensitivity: 71%, specificity: 79%). The most important parameter to predict the need for intensive care treatment was the neutrophil count, with an AUC of 0.782, sensitivity of 73%, and a specificity of 75% and a cutoff value of 4.43. Rizo-Téllez et al. [15] found an NMR >17.75 to be a good independent risk factor for predicting mortality with a sensitivity of 89.4% and a specificity of 80%. Peng et al. [16] found that NMR was significantly associated with the severity of COVID-19 (12.4 vs. 8.0 in severe and non-severe patients; p<0.001). In this study, the NMR value was found to be significant in both univariate and multivariate analysis as a predictor of the need for intensive care. ROC analysis with an NMR cutoff value of 17.6 had an AUC of 0.678, a sensitivity of 43%, and a specificity of 89%. The NMR was significantly greater in the ICU group in both univariate and multivariate analysis and was a predictor of mortality.

Many studies have suggested that hemoglobin, hematocrit, and RDW values may be independent risk factors associated with severe disease [17]. In a meta-analysis conducted by Lippi et al. [18], RDW was found to be useful for assessing the risk of unfavorable COVID-19 progression. It was reported in another study that an elevated RDW measured at admission and increasing RDW during hospitalization were associated with a significantly higher mortality risk [19]. Similarly, in this study, the RDW and MCV levels were significantly lower and the hemoglobin and hematocrit values were significantly higher in outpatients compared with the ICU and inpatient groups. With values adjusted for age and sex, univariate analysis re-

vealed lower hemoglobin and hematocrit values in the ICU group. However, the RDW was not significant in univariate or multivariate analysis of patient need for ICU care or as a predictor of mortality, nor did this parameter correlate with the length of hospitalization.

It was observed in a meta-analysis that evaluated 1779 patients that a low platelet count was associated with increased risk of severe disease and mortality [20, 21]. Furthermore, the MPV and PDW were higher in non-survivors on admission day in another study [8]. The MPV can provide important information about the course and prognosis in many pathological conditions [22]. This includes diseases such as cardiovascular diseases, respiratory diseases, Crohn's disease, rheumatoid arthritis, inflammatory disease such as juvenile systemic lupus erythematous, diabetes mellitus, and many neoplastic diseases [23-28]. It has been demonstrated that inflammatory cytokines regulate both prothrombotic and proinflammatory events by regulating thrombopoiesis and MPV. A high MPV is associated with cardio and cerebrovascular disorders and low-grade inflammatory conditions prone to arterial and venous thrombosis [29, 30]. It is thought that a high MPV, which is also associated with hypercoagulation and inflammation, may be associated with COVID-19 complications. The intensity of systemic inflammation is also correlated with different sized platelets [29]. In this study, there was no significant difference between groups in terms of platelets, MPV or PDW. However, univariate analysis indicated that the MPV and PDW were significant, while multivariate analysis yielded only the MPV as a significant predictor of the need for intensive care treatment. These parameters were not associated with mortality prediction or the length of hospitalization.

Wang [31] reported that the CRP level was an early parameter that indicated disease severity, and Yao et al. [32] noted that the D-dimer level correlated with disease severity and was a reliable prognostic parameter for in-hospital mortality. In a study of 4103 patients, it was found that age and the CRP and D-dimer levels were the strongest risk factors affecting hospitalization [33]. Tang et al. [34] found that an elevated D-dimer level was common in COVID-19 deaths, and Wang et al. [35] developed a laboratory model including age, neutrophil and lymphocyte counts, and CRP and D-dimer levels. We found that procalcitonin, CRP, and D-dimer values were related to disease severity. Procalcitonin, CRP, and D-dimer values were significantly higher in the ICU patients than the inpatients or outpatients ($p<0.001$). The procalcitonin, CRP, and D-dimer levels in inpatients were also higher than in the outpatients. They were significantly higher in the ICU patients than in the outpatients or the inpatients. They also were significant in univariate analysis to predict the need for intensive care. A D-dimer level >1000 (μg/L) and a CRP level >10 times the normal level are among the hospitalization criteria in the adult patient treatment guideline of the Turkish Ministry of Health. In this study, similar to other reports in the literature, age and CRP and D-dimer levels were observed to be the parameters most associated with the duration of hospitalization ($r=0.429$, 0.259, 0.368, respectively; $p<0.01$). The lymphocyte count was not associated with the duration of hospitalization.

Conclusion

There are many opinions in the literature about laboratory parameters and the prediction of mortality and severity in this new disease. Thus far, the most valuable parameter to predict the need for intensive care is the neutrophil count (AUC, sensitivity, and specificity of 0.782, 73%, and 75%, respectively), and the best predictive marker of mortality is the NMR and the procalcitonin value. Age, the MPV, and the procalcitonin, CRP, and D-dimer levels were found to be positively correlated with the length of hospital stay.

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

Ethics Committee Approval: This study was approved by the Health Sciences University Sisli Hamidiye Etfal Training and Research Hospital Clinical Research Ethics Committee (Date: 22.09.2020; No: 3002).

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Research Article

Apolipoprotein E and adiponectin levels in coronary artery disease patients with low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol discordance

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Abstract

Objectives: The aim of this study was to investigate whether serum apolipoprotein E (ApoE) and adiponectin were associated with discordance of low-density lipoprotein cholesterol (LDL-C) and non-high-density lipoprotein cholesterol (non-HDL-C) levels in coronary artery disease (CAD) patients.

Methods: A total of 243 patients with significant CAD were studied. The fasting levels of serum lipids, plasma ApoE, and adiponectin were measured. The median LDL-C and non-HDL-C values were determined to assess and analyze discordance. Discordance was defined as an LDL-C≥ the median and non-HDL-C< the median, or an LDL-C< the median and a non-HDL-C≥ the median. ApoE and adiponectin were compared between discordant and concordant groups.

Results: Discordance between the LDL-C and the non-HDL-C was observed in 14% of the patients. Although the median adiponectin was lowest in the group with an LDL-C< median and a non-HDL-C≥ the median, and the median ApoE was lowest in the group with an LDL-C≥ median and a non-HDL-C< the median, no statistically significant differences in the ApoE and adiponectin were recorded between the groups (p=0.186 and p=0.161, respectively). Adiponectin was negatively correlated with triglyceride and remnant cholesterol (p<0.001 and p<0.001, respectively).

Conclusion: While discordance between the LDL-C and the non-HDL-C in CAD patients (14%) was observed, the plasma ApoE and adiponectin levels were not significantly different between the discordant and concordant groups.

Keywords: Adiponectin, apolipoprotein E, coronary artery disease

Atherosclerosis is a multifactorial disorder and lipoprotein abnormalities are important in the pathogenesis of the disease. A subset of patients with either normal lipid levels or low levels of low-density lipoprotein cholesterol (LDL-C) following treatment still experiences atherosclerotic events [1]. Other hidden lipoprotein alterations may have a potential role in the pathogenesis of atherosclerosis.

Apolipoprotein E (ApoE), predominantly synthesized in the liver, is essential for lipid metabolism. It promotes the clearance of remnants of triglyceride-rich lipoproteins TRLs (TRLs) from the circulation into the liver [2]. ApoE is not a constituent of the LDL particle [3, 4]. ApoE, although generally considered antiatherogenic, is associated with metabolic syndrome and ApoE-rich lipoprotein particles are atherogenic [5-7].

Adiponectin is secreted from adipose tissue and displays antiatherogenic and anti-inflammatory properties [8, 9]. It is linked to obesity, diabetes mellitus and dyslipidemia [10-12].

LDL-C and non-high-density lipoprotein cholesterol (non-HDL-C) are main targets for lipid-lowering therapy to prevent atherosclerotic events. However, not all patients have concor-

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dant LDL-C and non-HDL-C levels. Reports have suggested that a sizeable proportion of patients have a low LDL-C and a high non-HDL-C level, or a high LDL-C and a low non-HDL-C level [13-15]. The objective of this study was to investigate whether the serum ApoE and serum adiponectin values were associated with discordance of the LDL-C and non-HDL-C levels in patients with significant coronary artery disease (CAD).

Materials and Methods

Study design and patients

This was a cross-sectional study. The research protocol was approved by Istanbul University Faculty of Medicine Clinical Research Ethics Committee on October 13, 2017 (no: 16). Written informed consent was obtained from each patient. Patients referred for coronary angiography with the suspicion of stable CAD were prospectively recruited between June 2018 and January 2019. The coronary angiography procedures were performed through the femoral artery using the standard Judkins method. The baseline diagnostic angiograms of the patients were assessed independently by 2 experienced interventional cardiologists who were blinded to the patients' lipid parameters, and ApoE and adiponectin levels. Epicardial coronary vessel stenosis of ≥50% was considered significant CAD. Diffusion of CAD lesions was assessed by calculating the Gensini score [16]. The exclusion criteria for the study were a history of coronary revascularization, severe valvular disease or heart failure, malignancy, inflammatory disease, active infection, renal or hepatic insufficiency, or hypo/hyperthyroidism. Since statins and peroxisome proliferator-activator receptor agonists have been suggested to effect plasma adiponectin and ApoE levels, patients using a statin, fibrate, or thiazolidinedione were also excluded [17-19]. In all, the study group comprised 243 patients with significant CAD detected during coronary angiography.

The clinical parameters assessed were age, gender, and coronary risk factors. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or current medication with antihypertensive drugs. Patients were classified as diabetic if they had been informed of this diagnosis prior to the study and had been using oral antidiabetic drugs or insulin treatment upon study admission. Body mass index (BMI) was calculated as body weight in kilograms divided by the squared height in meters (kg/m^2).

Laboratory measurements

Lipid measurements were performed using fasting blood samples taken before the angiography. The plasma concentration of total cholesterol, LDL-C, HDL-C, and triglycerides (TG) was measured with a clinical biochemistry analyzer (Architect c8000; Abbott Laboratories, Lake Bluff, IL, USA). The enzymatic colorimetric method was used for quantitative determination of total cholesterol. The endpoint colorimetric method was used for quantitative determination of HDL-C. LDL-C was measured using the quantitative colorimetric method. The glycerol phosphate oxidase method was used to determine a quantitative TG level measurement. The level of non-HDL-C was calculated as total cholesterol minus HDL-C and remnant cholesterol was calculated as non-HDL-C minus LDL-C.

The concentration of ApoE was assessed immunonephelometrically using a BN ProSpec analyzer (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Adiponectin was measured immuneturbidimetrically using a Randox kit (Randox Laboratories Ltd., Crumlin, County Antrim, UK) and a Cobas c501 chemistry analyzer (Roche Diagnostics, Basel, Switzerland).

Statistical analysis

Categorical variables were defined as percentages. The normality of the distribution of continuous variables was evaluated with the Kolmogorov-Smirnov test or the Shapiro-Wilk test. Continuous data were described as mean±SD for normal distributions, and median (interquartile range [IQR]) for skewed distributions. Pearson's or Spearman's correlation analysis was used to examine the correlation between continuous variables of lipid parameters, ApoE, and adiponectin. Differences between the 2 groups were analyzed using the Mann-Whitney U test. First, the medians for LDL-C and non-HDL-C we determined in order to examine the discordance between them. The patients were categorized into groups of less than or equal to/greater than the median LDL-C and non-HDL-C levels. As there is no standard cutoff point for discordance, the median was selected to define discordance and to make it easier to apply to our study population. Discordance was defined as an LDL-C≥ the median, and non-HDL-C as < the median, or an LDL-C< the median and a non-HDL-C≥ the median. Concordant groups were defined as both the LDL-C and the non-HDL-C≥ the median, or both LDL-C and non-HDL-C< the median. Differences between the baseline patient characteristics across these categories were analyzed with a chi-squared test to compare categorical variables and one-way analysis of variance or the Kruskal-Wallis test for continuous measures. The Bonferroni or Conover-Inman test was performed for binary comparisons between the groups. The data analysis was performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). A p value <0.05 was accepted as statistically significant.

Results

The mean age of the study population was 61.4±11.6 years and 53.9% of the 243 patients were male. The baseline characteristics are presented in Table 1. The mean serum adiponectin concentration was 7.3±3.9 µg/mL and the mean serum ApoE concentration was 3.9±2.8 mg/dL.

The LDL-C levels were strongly and positively correlated with the non-HDL-C levels (r=0.849; p<0.001), but there was

Table 1. Baseline characteristics of the study population

Characteristics	
Gender (% male)	53.9
Age (years) (mean±SD)	61.4±11.6
Smoking (%)	41.6
Hypertension (%)	45.3
Diabetes (%)	36.3
Body mass index (kg/m^2) (mean±SD)	23.5±3.8
Biochemical analysis (mean±SD or median [interquartile range])	
Total cholesterol (mg/dL)	198.2±47.4
LDL-C (mg/dL)	117 (51)
HDL-C (mg/dL)	40.7±9.5
Triglycerides (mg/dL)	140 (95)
Non-HDL-C (mg/dL)	157.4±45.5
Fasting glucose (mg/dL)	118.9±38.7
Creatinine (mg/dL)	0.92±0.38
Mean Gensini score	35.6±11.1
Mean adiponectin (µg/mL)	7.3±3.9
Mean ApoE (mg/dL)	3.9±2.8

ApoE: Apolipoprotein E; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

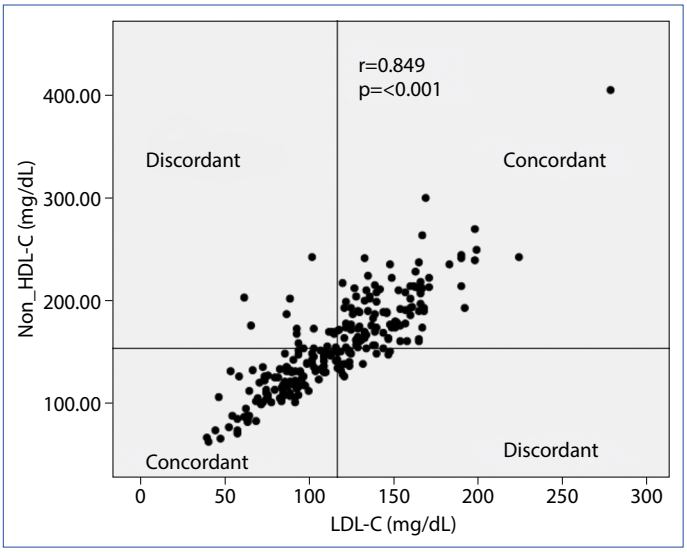

Figure 1. Scatterplot and prevalence of discordance and concordance defined according to median values of LDL-C and non-HDL-C.

HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

discordance between them. Discordance of LDL-C and non-HDL-C values was found in 14% of the patients. The magnitude of the discordance and the distribution of LDL-C and non-HDL-C levels according to median values are shown in Figure 1. Non-HDL-C was positively correlated with TG (r=0.474; p<0.001).

Adiponectin was negatively correlated with both TG and remnant cholesterol (r=-0.299; p<0.001 and r=-0.259; p<0.001, respectively). It was positively correlated with HDL-C (r=0.227; p<0.001). Adiponectin was not correlated with LDL-C or non-HDL-C (p=0.081 and p=0.815, respectively). ApoE was not correlated any of the lipid parameters. Furthermore, adiponectin was not correlated with ApoE (p=0.838). When the patients were divided into 2 groups according to HDL-C level, the patients with an HDL-C ≥40 mg/dL had higher adiponectin levels than patients with an HDL-C <40 mg/dL (6.8 [IQR: 5.5] µg/mL and 4.8 [IQR: 4.7] µg/mL, respectively; p<0.001). Patients with a TG level ≥ 200 mg/dL had lower adiponectin levels than patients with a TG <200 mg/dL (3.9 [IQR: 3.2] µg/mL and 6.6 [IQR: 5.5] µg/mL, respectively; p<0.001). Female patients with an HDL-C ≥50 mg/dL had higher adiponectin levels than female patients with an HDL-C <50 mg/dL (7.8 [IQR: 6.5] µg/mL and 5.9 [IQR: 5.4] µg/mL, respectively; p<0.038).

To further evaluate the characteristics of patients with discordance and concordance of LDL-C and non-HDL-C, the patients were classified into 4 subgroups: Group 1: LDL-C< the median and non-HDL-C< the median, group 2: LDL-C< the median and non-HDL-C≥ the median, group 3: LDL-C≥ the median and non-HDL-C< the median, and group 4: LDL-C≥ the median and non-HDL-C≥ the median. Groups 2 and 3 were discordant (Table 2). The variables of age, gender, BMI, smoking history, and the percentage of patients with hypertension were not significantly different between the groups. The patients in group 2 (LDL-C< the median and non-HDL-C≥ the median) had the highest prevalence of diabetes mellitus (p=0.007). Although the median adiponectin measurement was lowest in group 2, the TG levels were highest, and the median ApoE was lowest in group 3 (LDL-C≥ the median and non-HDL-C< the median), there were no statistically significant differences between groups in the ApoE or adiponectin levels (p=0.186 and p=0.161, respectively).

Discussion

In the present study, we assessed the cross-sectional association between LDL-C and non-HDL-C discordance and the serum adiponectin and ApoE concentrations in patients with significant CAD. The serum levels of adiponectin and ApoE in the discordant and concordant groups were not significantly different.

A sizeable proportion of patients have discordant LDL-C and non-HDL-C. The discordance rate in our study was 14%, which is similar to that of previous studies [13-15]. It has been suggested that the LDL-C alone may give a false sense of security in one-fifth of diabetic subjects with a low LDL-C level, and patients with a normal LDL-C level may still be at risk for cardiovascular events [20]. Non-HDL-C is the second target for lipid-lowering therapy and represents the cholesterol content of all circulating atherogenic lipoproteins [21]. It has been demonstrated that discordance of LDL-C and non-HDL-C either underestimates or overestimates coronary risk [14]. In particular, studies that follow up with patients who have a low LDL-C and

Table 2. Characteristics of patients with concordant and discordant LDL-C and non-HDL-C levels

	LDL-C<median, non-HDL-C<median n=102 (group 1)	LDL-C<median, non-HDL-C≥median n=16 (group 2)	LDL-C≥median, non-HDL-C<median n=19 (group 3)	LDL-C≥median, non-HDL-C≥median n=106 (group 4)	p
Age (years)	63.4±12.4	58.7±11.3	61.9±10.5	59.6±10.7	0.085
Gender (% male)	58.8	56.3	47.4	50	0.571
Smoking (%)	43.1	50.0	42.1	39.6	0.877
Hypertension (%)	52.9	50.0	42.1	36.8	0.127
Diabetes (%)	40.2	68.8	15.8	32.1	0.007
Body mass index (kg/m^2)	23.6±3.8	24.2±4.4	24.2±3.4	23.2±3.9	0.620
Total cholesterol (mg/dL)	164 (34)	204.5 (31)	189 (16)	230 (36)	<0.001[a,b,c,e,f]
LDL-C (mg/dL)	86.5 (25)	99.5 (22)	121 (7)	145 (31)	<0.001[a,b,c,d,e,f]
HDL-C (mg/dL)	40 (17)	32.5 (7)	45 (12)	42 (9)	<0.001[a,d,e]
Triglycerides (mg/dL)	116 (80)	275 (184)	104 (50)	164 (97)	<0.001[a,c,d,e,f]
Non-HDL-C (mg/dL)	121 (28)	170 (27.5)	143 (13)	190 (41)	<0.001[a,c,d,e,f]
Fasting glucose (mg/dL)	109 (34)	112 (96)	111 (17)	100(37)	0.217
Gensini score	36.1±17.6	45.8±11.4	23.2±18.1	35.3±20.1	0.856
Adiponectin (µg/mL)	5.2 (4.2)	4.7 (2.8)	9.1 (5.2)	6.6 (5.6)	0.161
ApoE (mg/dL)	2.4 (6.1)	3.4 (5.5)	1.0 (3.3)	2.7 (5.8)	0.186

Data are expressed as percentage for categorical variables; chi-squared test was used. Data are presented as mean±SD or median (interquartile range) for continuous variables, and one-way analysis of variance or Kruskal-Wallis test were used to compare continuous measures. Statistically significant p values are in bold. The Bonferroni or Conover-Inman test was performed for binary comparisons between groups, and the p value was set at 0.05. Significant differences were found between a) group 1 vs group 2, b) group 1 vs group 3, c) group 1 vs group 4, d) group 2 vs group 3, e) group 2 vs group 4, and f) group 3 vs group 4. ApoE: Apolipoprotein E; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

a high non-HDL-C or vice versa and evaluate the impact of discordance on a CAD prognosis are currently lacking.

Discordance of LDL-C and non-HDL-C has been associated with obesity, glucose abnormalities, hypertriglyceridemia, and low HDL levels in previous studies [14, 15]. Adiponectin and ApoE levels have also been found to be associated with similar risk factors. A low plasma concentration of adiponectin has been observed in patients with metabolic syndrome, obesity, and diabetes mellitus [10, 11]. ApoE levels increased in parallel with the number of metabolic syndrome components [6]. Both ApoE and adiponectin were associated with CAD and serum lipids. To further investigate the differences in CAD patients with discordant and concordant LDL-C and non-HDL-C, we evaluated plasma the ApoE and adiponectin concentrations.

Among lipoprotein abnormalities, a high LDL-C is major predictor of atherosclerosis, but many other hidden lipoprotein alterations may have a potential role in the pathogenesis of atherosclerosis. In a study by Barbagallo et al. [7], it was reported that patients with CAD had a higher concentration of ApoE-enriched TRLs than controls, despite both groups having similar TG, LDL-C, and non-HDL-C levels. The results of another study revealed that CAD patients had a lower concentration of ApoE-containing HDL than the subjects without CAD, though both had similar total cholesterol and TG levels [22].

Plasma lipids affect the concentration of plasma ApoE. In normolipidemic subjects, the majority of serum ApoE resides in the HDL, whereas in hypertriglyceridemic subjects, it resides in the TRLs [4]. Total ApoE, TRL-ApoE and intermediate-sized remnant-like lipoprotein-ApoE values were found to be higher in patients with hyperlipidemia than normolipidemic subjects [4]. The serum total ApoE concentration was significantly higher in low HDL-C subjects in a study that assessed ApoE at the extreme ends of HDL-C distribution [6]. We evaluated the serum ApoE concentration in patients with CAD and we further divided the patients into subgroups of those with discordant or concordant LDL-C and non-HDL-C. CAD severity was measured with the Gensini score and it was observed that the serum ApoE levels and the Gensini score did not differ significantly between the discordant and concordant groups. The ApoE value was numerically lowest (not statistically significant) in the group with the highest HDL-C and the lowest TG, but it was numerically highest in the group with the highest non-HDL-C and the highest LDL-C, rather than the group with the lowest HDL-C and the highest TG measurement.

Experimental studies have shown that adiponectin demonstrated antiatherosclerotic properties [23]. Also, a low serum adiponectin level has been associated with an increased risk of CAD in initially healthy subjects [24, 25]. In contrast to studies including healthy subjects, a high serum adiponectin level has been associated with increased mortality in patients with manifest CAD in many studies, including a meta-analysis [26-28]. The underlying mechanism of the adiponectin paradox in experimental studies of patients with and without CAD is still to be fully understood. Adiponectin has also been linked to

dyslipidemia. A high plasma concentration of adiponectin has been associated with increased HDL-C, increased apolipoprotein A1, and total cholesterol [12, 27, 29]. Moreover, it has been inversely correlated with serum TG and apolipoprotein B [12, 27, 29]. While the relationship between adiponectin and LDL-C is unclear, adiponectin has been found to be associated with smaller LDL particle size [30, 31]. In the present study, consistent with earlier studies, patients with a low HDL-C or high TG level had lower adiponectin levels than patients with a high HDL-C or a low TG level. The median LDL-C and non-HDL-C cutoffs used in our study indicated that the adiponectin was no longer significantly different between the discordant and concordant groups. It was numerically lowest (not statistically significant) in the discordant group with the lowest HDL-C and the highest TG.

Study limitations

This study has several limitations, such as the lack of measurement of ApoE subfractions or various isoforms of adiponectin. In addition, the plasma TRLs may or may not have contained ApoE, and the TRL or HDL may have been ApoE-poor or ApoE-rich. ApoE subfractions may differ in discordant and concordant groups. There is no absolute definition or standard cutoff value for the discordance of LDL-C and non-HDL-C. We used the median values of our study population. Other limitations include the small sample size and multiple factors that can affect plasma adiponectin levels and lipoprotein metabolism, including gender, age, dietary factors, and lifestyle. Age and gender did not differ between the discordant and concordant groups, but we did not assess dietary factors or lifestyle parameters. Considering the unmeasured potentially confounding factors in the population studied, the findings of our study should be interpreted as suggestive and additional, large-scale studies would be required to validate our results.

Conclusion

Among patients with significant CAD, 14% demonstrated a discordance of LDL-C and non-HDL-C. While high risk features, such as diabetes mellitus, hypertriglyceridemia, a higher total cholesterol and a higher LDL-C level differed between the discordant and concordant groups, the ApoE and adiponectin values were not significantly different. Adiponectin was negatively correlated with TG and positively correlated with HDL-C.

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

Ethics Committee Approval: The research protocol was approved by Istanbul University Faculty of Medicine Clinical Research Ethics Committee on October 13, 2017 (No: 16).

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Research Article

Association between *HBA* locus copy number gains and pathogenic *HBB* gene variants

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Abstract

Objectives: Alpha (α) and beta (β) thalassemia are the most prevalent genetic hematological disorders. The co-occurrence of silent β-thalassemia with excess α-globin gene copies is associated with the thalassemia intermedia phenotype. This study was an investigation of the α-globulin gene dosage and sequence variations in thalassemia patients.

Methods: Multiplex ligation-dependent probe amplification and Sanger sequencing were used to identify the hemoglobin subunit alpha 1 (*HBA1*) and *HBA2* gene alterations in 32 patients. Deletion, duplication, and other findings were analyzed in the index cases and family members.

Results: Four of the 32 cases (12.5%) were found to have gross duplications. Two cases demonstrated α-globin triplication, and 2 had a quadruplicated *HBA1/2* genes. Affected family members revealed genotype-phenotype correlation. In 1 patient, it was observed that quadruplicated *HBA* genes co-occurrence with hemoglobin subunit beta (*HBB*) mutation was inherited from his mother. Notably, the mother did not demonstrate any thalassemia phenotype. Further investigation showed that the mother was carrying a single copy *HBA* gene deletion in the trans allele that explained her clinical condition.

Conclusion: This study examined the effect of increased copies of the *HBA* gene in *HBB* gene pathogenic variant carriers. The results indicated that β-thalassemia mutations with a co-occurrence of increased α-globin gene dosage is not very rare condition. Patients with clinical findings incompatible with their *HBB* genotypes should be investigated for small and gross α-globin gene variants in order to provide genetic counseling and prenatal diagnosis follow-up, as appropriate.

Keywords: Alpha-globin gene quadruplication, co-inheritance of *HBA* and *HBB*, multiplex ligation-dependent probe amplification, thalassemia intermedia

Alpha (α) and beta (β) thalassemia, blood disorders characterized by decreased hemoglobin production, are the most frequently observed genetic disorders in the world. The carrier frequency has been found to be high in populations where malaria is common [1-3]. Cross-national migration from endemic regions has led to spread of the disease [4, 5]. Thalassemia is classified into 2 types according to the defective gene: In α-thalassemia, the hemoglobin subunit alpha (*HBA*) gene cluster is damaged, and in cases of β-thalassemia, there are pathogenic variants in the hemoglobin subunit beta (*HBB*) gene [6]. According to the globin mutation database, 940 point variants have been recorded that can lead to structural alterations, protein truncation, defective splicing, decrease in transcription, or loss of stability in the *HBB* gene [7]. The database also includes recordings of gross variants of *HBA1/2* genes, which are identified in 2% of thalassemia cases [8]. Four alleles of 2 structurally similar genes, *HBA1* and *HBA2* are responsible for α-globin gene expression and are critically important when establishing genotype-phenotype relationships in patients with β-thalassemia. Although the *HBA1* gene

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has 349 reported small sequence variants (also called non-deletion variants) reported in the globin mutation database and the *HBA2* gene has 455, large deletions are responsible for about 85% of patients with α-thalassemia [7]. Four phenotypes have been identified: hemoglobin Barts hydrops, with a loss of all 4 α-globin genes (--/--), which is usually fatal at birth; hemoglobin H (HbH) disease, with 3 missing α-globin genes (--/-α), which generally includes only moderate symptoms and may not require clinical intervention; α-thalassemia trait/carrier, with 2 absent or inactive *HBA* genes (-α/-α or - -/αα), which may result in very mild anemia; and α-thalassemia silent carrier, caused by the deletion of a single *HBA* gene (-α/αα), which is asymptomatic [9-11].

Although non-deletion variants of α-globulin genes are rarely seen in α-thalassemia, the HbH phenotype occurring with the homozygous form of α-thalassemia ($\alpha^{constant\ spring}$ α/-$\alpha^{constant\ spring}$) results in a truncated *HBA2* messenger-type RNA nonsense mutation in *HBA2* and leads to a more severe clinical course than a deletion HbH phenotype and requires transfusions [10-12]. β-thalassemia is classified into 3 types: thalassemia major, thalassemia intermedia, and thalassemia minor. The type is based on the nature of the mutation, protein function (β^0 or β^+), and zygosity, and is reflected in the severity of clinical symptoms. Heterozygous carriers are usually moderately anemic or healthy, though a few exceptions have been reported.

In addition to moderate/mild homozygous variations, compound heterozygotes or an elevated fetal hemoglobin level can influence the clinical picture [13-15]. Co-existing α-thalassemia and β-thalassemia is most frequently reported in Asian countries, which have a high prevalence of carriers of both α- and β-thalassemia [16-19].

Genotype-phenotype discordance has been reported in the literature in β-thalassemia intermedia and β-thalassemia minor cases. Inconsistency in hematological findings and β-thalassemia traits may be observed in circumstances such as iron deficiency, co-inheritance of α- and β-thalassemia variant heterozygosity, co-inheritance of *HBB* variations, and other globin gene variants [13]. Hepatitis, folic acid or vitamin B12 deficiency can also cause upregulation of α-thalassemia genes, leading to difficulties in interpreting genotype. The coupling of α-thalassemia alterations with *HBB* mutations may affect α/β globin ratio [20, 21]. In heterozygous *HBB* variant carriers, the phenotype may become more severe if there are also gains in α globin. On the contrary, in homozygous or compound heterozygous *HBB* variant carriers, the clinic may be milder if there is also a loss in *HBA* genes [22].

This study examined the effect of increased copies of the *HBA* genes in *HBB* gene pathogenic variant carriers.

Materials and Methods

This study was approved by the Istanbul University İstanbul Faculty of Medicine Ethics Committee on January 28, 2021 (no: 52410). The study cohort comprised 32 patients with hematological findings of thalassemia who were referred from the Istanbul Faculty of Medicine Pediatric Hematology Unit to the Istanbul Faculty of Medicine Department of Medical Genetics for genetic testing and counseling. Detailed pedigrees of the patients were recorded, medical findings were reviewed, and peripheral blood samples were collected upon receipt of written, informed consent.

DNA isolation was performed using a nucleic acid extraction kit (MagNA Pure; Roche Diagnostics, Basel, Switzerland). Primers were designed in-house for the *HBA1* (NM_000558.5), *HBA2* (NM_000517.6), and *HBB* (NM_000518.5) genes for Sanger sequencing using a genome analyzer (ABI3500). Deletion and duplication analysis was performed using multiplex ligation-dependent probe amplification (MLPA) (MLPA Probemix P140 *HBA*; MRC-Holland bv, Amsterdam, The Netherlands) and analyzed with Coffalyser.Net MLPA analysis software (MRC-Holland bv, Amsterdam, The Netherlands).

Results

In all, 4 index patients and family members were identified as variant carriers of the *HBB* gene with an increased *HBA* copy number. The mean hematological data was red blood cell count: 5.3x10^6±1.4/mm^3 (range: 3.9-7.2x10^6/mm^3), mean corpuscular volume (MCV): 59.8 fL±7.6 (range: 55.1-71.1 fL), mean corpuscular hemoglobin (MCH): 18.7±2.6 pg (range: 17.1-2.26 pg), Hb: 8.2±0.9 g/dL (range: 7.2-9.2 g/dL), HbA 87.4±7.2% (range: 76.7-92.3%), and HbA2: 4.9±1.7% (range: 2.6-6.7%).

The hematological and molecular findings of the study cases and family members are presented in Figures 1-4. In Family 1, the index case and affected sister were found to have ~81Kb of heterozygous quadruplication in the α-globin re-

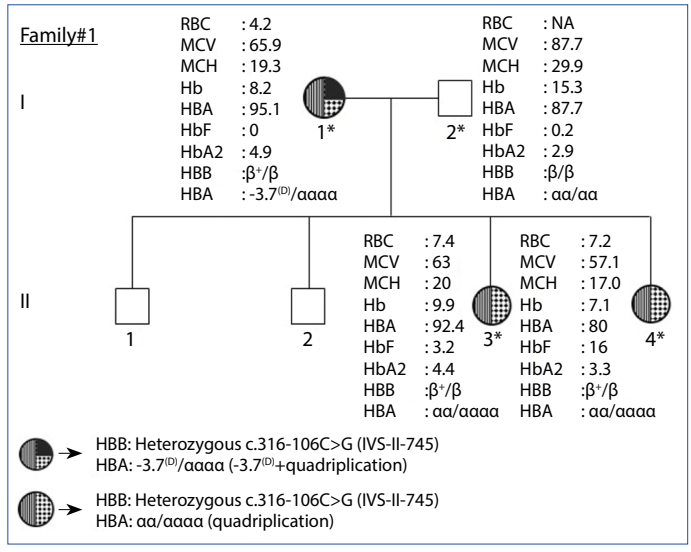

Figure 1. Genetic and hematological results of Family 1.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

gion (beginning in the HS40 element) with β^+ associated heterozygous c.316-106C>G (IVS-II-745, rs34690599) in the *HBB* gene. In addition to these 2 mutations, their mother had an *HBA* -3.7$^{(D)}$ heterozygous deletion in the other *HBA* allele. The father's results were normal (Fig. 1). In the index case of Family 2, an *HBB* heterozygous c.92+1G>A (IVS-I-1, rs33971440)

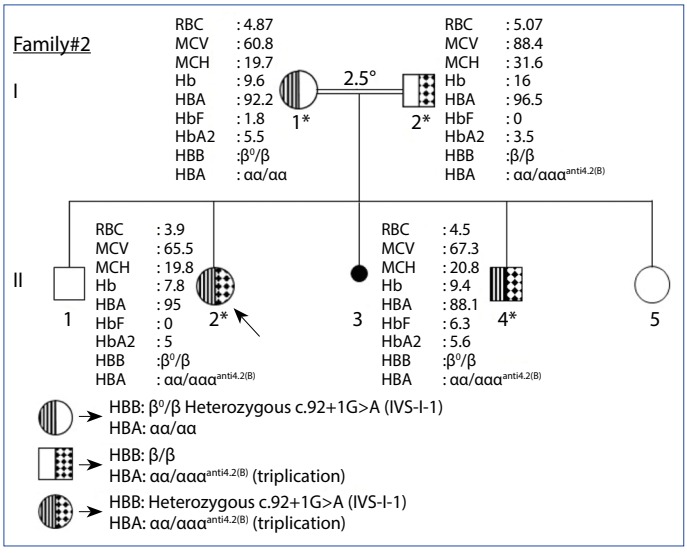

Figure 2. Genetic and hematological results of Family 2.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

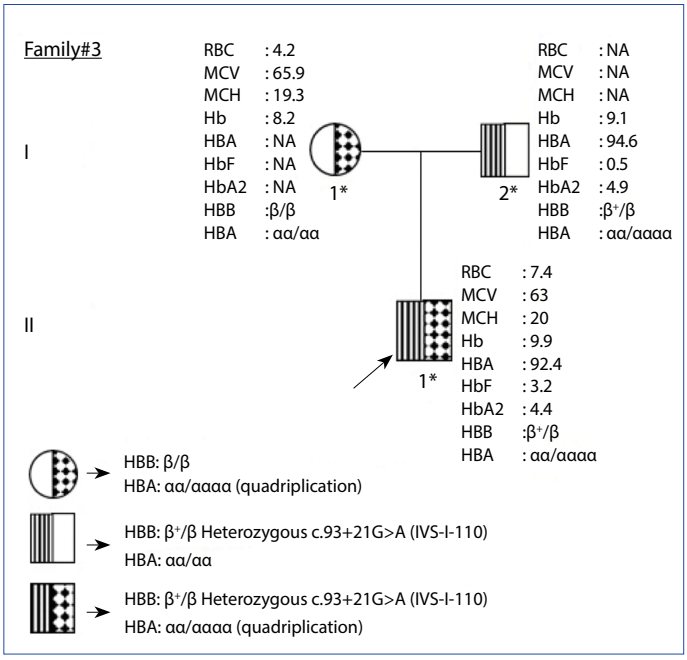

Figure 3. Genetic and hematological results of Family 3.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

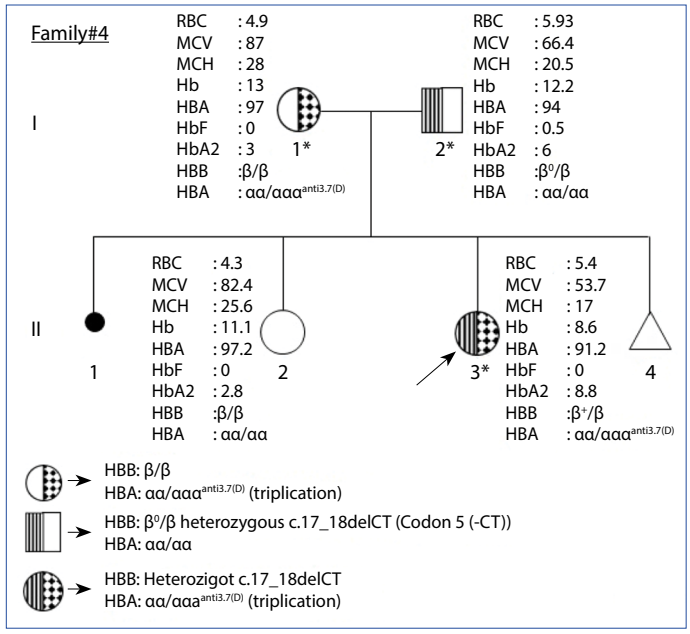

Figure 4. Genetic and hematological results of Family 4.

Hb: Hemoglobin; HBA: Hemoglobin subunit alpha; HbA2: Hemoglobin subunit alpha 2; HBB: Hemoglobin subunit beta; HbF: Fetal hemoglobin; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; RBC: Red blood cell count.

mutation and ααα$^{anti4.2(B)}$ were detected. The affected brother had a similar genotype, while the other siblings had inherited the *HBB* mutation from the mother and *HBA* triplication from the father (Fig. 2). In Family 3, heterozygote c.93-21G>A (IVS-I-110, rs35004220) in the *HBB* gene and ~81Kb of heterozygous quadruplication (beginning in the HS40 region) were detected in the *HBA* locus of the index case. Investigation of the family showed that the patient had inherited both digenic alterations from the father (Fig. 3). The index case of Family 4 carried a heterozygous c.17_18delCT (p.Pro6fs, rs34889882) inherited from the *HBB* gene of his father and ααα$^{anti\ 3.7(D)}$ from his mother (Fig. 4).

Discussion

Co-inheritance of β-thalassemia and α-thalassemia is frequently seen in populations where carriers of both α- and β-hemoglobinopathy are prevalent [16, 23]. Cases with co-inheritance of β-thalassemia with α-globin gene deletion typically are presented with a milder phenotype. This is a result of a decrease in α/β globin ratio that is higher in β-thalassemia cases carrying a normal copy number of *HBA* genes [22, 24]. It has been reported that the coexistence of *HBB* mutations and *HBA1/HBA2* gene triplication or quadruplication results in a more severe phenotype since the ratio of α/β is predicted to deteriorate as the α-globin dose increases [18, 25-33]. In the present study, we found 4 β-thalassemia carrier cases with an α-globin gene copy gain and an unusual phenotype. Each member of the families had an intrafamilial genotype-phenotype correlation. Cebrian et al. [34] reported a milder pheno-

type in heterozygous *HBB* carriers than homozygous carriers among members of a family carrying *HBB* c.93-21G> A (β^+) and *HBA* triplication. In a study of 6 families, Origa et al. [35] reported the need for transfusions in 4 of 14 individuals with a quadruplicated α-globin gene and *HBB* heterozygotes. Other family members had milder phenotypes, despite carrying the same genotype as a result of other genetic factors. Similarly, Farashi et al. [25] observed a thalassemia intermedia phenotype in a patient with heterozygous β-thalassemia found to carry 6 α-globin gene copies. The daughter of this patient had the same *HBB* genotype but 7 copies of α-globin and a clinically similar phenotype to her father.

The fact that our patients with triplication and quadruplication of the α-globin gene and mono-allelic *HBB* mutations had a moderate-to-severe thalassemia intermedia phenotype was consistent with the literature. Our study findings similarly supported reports that α/β globin ratios influence the severity of the disease [17, 22, 36]. In Family 1, the mother's heterozygous *HBB* mutation and *HBA* gene deletion/quadruplication in trans form resulted in a milder phenotype than the index. This case is a good example of why even silent *HBA* alterations should be investigated for segregation to reveal genotype-phenotype variation in the family. Expansion of the segregation may further aid our understanding of the effect of variants in Families 2, 3, and 4, in which *HBB* and *HBA* genes variants were found to inherited bi-parentally. While the parent with the *HBB* mutation alone demonstrated classic β-thalassemia minor, no clinical consequence was revealed in the parents with only α-globin gains. The β^+ *HBB* mutation in our 2 families with triplication and β^0 in the *HBB* mutations in the families with quadruplicated *HBA* prevented comparison of the copy number effect in those families. However, all of the index cases and affected siblings had similar genotypes compatible with thalassemia intermedia. In Family 3, detailed analysis was not possible because the only hematological information for the index patient's father that was available was the molecular results.

Non-allelic homologous recombination is the mechanism for the formation of deletions in α-thalassemia. One product of this recombination is deletion, however, duplication (triplication or quadruplication) is also possible [37, 38]. The frequency of duplication and deletion should be similar according to the mechanism. Though it is thought that there should be more carriers of duplication, since it does not cause mortality and morbidity, as deletions can, α-globin gene deletions have a heterozygous advantage over the malaria parasite, and over time the deletions have become more frequent [1, 39]. This may be why *HBA* deletions are observed more often in *HBB* carriers than duplications. Wu et al. [40] reported that triplications in southern China were 3 times more frequent than quadruplication. Frequent observance of both α- and β-thalassemia mutations in geographical regions where hemoglobinopathies are common leads to the formation of complex thalassemia, resulting in phenotypic findings. Individuals who do not carry the *HBB* mutation but carry α-globin gene duplication are phenotypically normal. Therefore, these duplications are typically only revealed incidentally [35].

HBA studies in Turkey have been performed using restriction enzyme and gap- polymerase chain reaction methods for many years [41, 42]. Target-specific reverse dot blot kits (strip assay) have also been used [43-45]. However, since studies with these widely used kits were limited to targeted regions, only αααanti-3.7 increases from α-globin gains could be investigated. In recent years, the widespread use of MLPA studies has allowed for the identification of a wide spectrum of deletions and duplications in the *HBA* locus [8, 46]. Our study is important in that α-globin dosage increases in *HBB* heterozygous carriers revealed atypical clinical findings.

Children, born to parents one carrying *HBB* and the other *HBA* mutation or multiplication (triplication, quadruplication, etc.), have an increased risk for thalassemia intermedia. Farashi et al. [47] reported that co-inheritance of *HBB* carriers and α-globin gene multiplications was as high as 25% in some parts of Iran. Therefore, prenatal *HBA* analysis should be performed for *HBB* carriers. In this study, *HBA* gains were detected in 4 of 32 families (12.5%) with atypical clinical findings of *HBB* mutation. Co-inheritance of *HBA* gains and *HBB* mutations is not very rare in our population. Low MCV and MCH values in *HBA*-deletion carriers compared with normal individuals suggest the need for *HBA* molecular tests. However, since α-globin dose increases do not prompt a clinical finding in carriers, they pose a risk for the fetal phenotype in prenatal diagnosis and create difficulty in genetic counseling. The study of possible *HBA1/HBA2* gene mutations and dosage increases in β-thalassemia cases with phenotypes that do not match clinical expectations is an auxiliary factor in prenatal diagnosis and genetic counseling to identify the risk of inherited hemoglobinopathy and making informed decisions.

Conclusion

According to the data obtained in this study, *HBA* gene increases can be detected in *HBB* cases showing genotype-phenotype discordance at a rate that cannot be ignored. The detection of carrier families and individuals will contribute to accurate and effective guidance and genetic counseling in prenatal diagnosis or PGD studies.

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Conflict of Interest: The authors declare that there there is no conflict of interest regarding the publication of this article.

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Research Article

Assessment of analytical process performance using the Six Sigma method: A comparison of two biochemistry analyzers

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Abstract

Objectives: Six Sigma is a method of quality management analysis that integrates accuracy and precision of measurement, error identification, and process improvement. The aim of this study was to evaluate the analytical process performance of routine biochemical tests performed with 2 biochemistry analyzers in our laboratory according to Six Sigma methodology and compare the findings.

Methods: Internal quality control (IQC) data of routine biochemical analytes used for 3 months in 2 Abbott Architect c16000 analyzers (Abbott Diagnostics Inc., Lake Forest, IL, USA) were extracted and the mean, SD, coefficient of variation %, bias % and sigma values were calculated. The performance of the analytes was classified according to the sigma level: <3 demonstrated poor performance, 3-6 was graded as acceptable, and >6 indicated good performance.

Results: For both analyzers, 2 levels IQC sigma values of chloride and sodium were <3, while the levels of alkaline phosphatase, aspartate aminotransferase, amylase, creatine kinase, iron, gamma-glutamyl transferase, and magnesium were >6; and the sigma values of total bilirubin, phosphorus, glucose, high-density lipoprotein-cholesterol, total cholesterol, calcium, creatinine, and total protein were determined to be within the acceptable range of 3-6. Amylase and creatine kinase were the best performers on both analyzers, while sodium had the lowest sigma values.

Conclusion: Six Sigma is a good method to evaluate the analytical process performance of a clinical laboratory. Quality control measures should be implemented for parameters with low sigma values.

Keywords: Laboratories, quality control, six sigma

Clinical laboratory reports play a critical role in clinical decisions about patients. Therefore, clinical laboratories should evaluate process performance and minimize laboratory errors in order to produce the most accurate and reproducible test results possible. There are 3 basic stages in the total test process of medical laboratories: pre-analytical, analytical, post-analytical. It has been reported that 30% to 75% of laboratory errors occur in the pre-analytical phase, 9% to 55% in the post-analytical phase, and 4% to 30% in the analytical phase [1].

Laboratories should evaluate their process performance according to scientifically accepted quality criteria. This assessment includes the percentage of sample errors and rejections in the pre-analytical phase, the accuracy and precision measurement of test results in the analytical phase, and critical values reporting and test turnaround times in the post-analytic phase [2].

Clinical laboratories approve the validity of the analysis process according to quality control procedures for each analyte. Quality control consists of internal quality control (IQC) and external quality control (EQC) measures. IQC generally employs 2 or 3 levels of clinical decision points and daily IQC results are interpreted using control charts, such as the Levy-Jennings and Westgard rules. EQC samples are provided to clinical chemistry laboratories by an external agency once a month for use in analyzing and reporting [1].

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Analytical process errors comprise systematic and random errors that have basic parameters such as inaccuracy and imprecision. These parameters are expressed as bias and coefficient of variation (CV), respectively. Total error (TE) can be calculated using bias and CV for each test (TE=Bias+1.65CV) [3]. The allowable total error (TEa) is a parameter provided by reports such as the US Clinical Laboratory Implementation Amendments 1988 (CLIA'88) and the German RiliBÄK [4, 5]. Evaluation of the process performance of a clinical laboratory is essential for comparison with laboratories around the world and to ensure high quality standards. During the analytical phase, variables can be assessed according to quality control and calibration procedures [6]. Analytical process performance can be evaluated using process sigma levels, quality indicators, and patient test results [7].

Six Sigma is a quality management method that integrates accurate and precision evaluation, error identification, and process improvement. The Six Sigma method has been used in hospital quality management since 1999 [8]. The universal application steps are to define, measure, analyze, develop, and control. The sigma value can be calculated by laboratories using the TEa and bias and CV % levels [sigma=(TEa %-bias %) /CV %]. A higher sigma level reflects greater consistency and stability of laboratory tests. A low sigma value indicates poor quality, defined as defects per million opportunities (DPMO). The process sigma values according to DPMO recorded in this study are shown in Table 1 [9].

Bias and SD values, which are the criteria of accuracy and repeatability, are obtained from IQC or EQC programs regularly used in clinical laboratories. While some studies suggest calculating the bias values using IQC data, others recommend using EQC data [1, 10-12].

In this study, the analytical process performance of routine biochemical tests performed using 2 biochemistry analyzers in our laboratory was evaluated using Six Sigma methodology and compared.

Materials and Methods

The present study was conducted between January 1, 2020 and March 30, 2020 in the clinical chemistry laboratory of Adiyaman University Research and Education Hospital. The IQC data of 2 Abbott Architect c16000 analyzers (Abbott Diagnostics Inc., Lake Forest, IL, USA) were extracted for the following parameters: albumin (Alb), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), amylase (AMY), direct bilirubin (DBIL), total bilirubin (TBIL), creatine kinase (CK), iron (Fe), phosphorus (P), gamma-glutamyl transferase (GGT), glucose (Glc), high-density lipoprotein-cholesterol (HDL-Cholesterol), total cholesterol, calcium (Ca), chloride (Cl), creatinine (CREA), lactate dehydrogenase (LD), lipase, magnesium (Mg), potassium (K), sodium (Na), total protein (TP), triglyceride (TG), uric acid (UA), and urea. The daily level 1 (normal concentration) and level 2 (abnormal concentration) IQC material used was Technopath MultiChem S Plus (Lot: 18609180; Technopath Life Sciences, Ballina, Co. Tipperary, Ireland) and was applied according to the manufacturer's instructions.

Table 1. Process sigma level according to defects per million opportunities

Sigma level	DPMO
1	691462
2	308538
3	66807
4	6210
5	233
6	3.4

DPMO: Defects per million opportunities.

IQC level 1 (n=65 for each test) and IQC level 2 (n=65 for each test) data were obtained from the laboratory information system. The target mean values of the IQC material were those specified by the IQC material manufacturer and the target TEa levels were determined according to the CLIA'88. The CLIA total error criteria can be freely accessed at http://www.westgard.com.

The laboratory mean, SD, CV %, bias %, and sigma values were calculated for all of the analytes and the performance was graded according to the sigma level (<3: poor performance, 3-6: acceptable, >6: good performance).

The statistical analysis was performed using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA). Non-acceptable control data were excluded from the study. The mean, SD, CV %, bias %, and sigma values of the acceptable control data were calculated using the following the formulas:

CV %=SD/Mean×100

Bias %=(Laboratory mean-Target Mean)/Target Mean×100

Sigma=(TEa-Bias)/CV

Results

The target mean, TEa level, laboratory mean, SD, CV %, bias %, and sigma values for IQC level 1 and level 2 of the tests run on the first Abbott Architect c16000 analyzer are shown in Table 2, and results for the second Abbott Architect c16000 analyzer are shown in Table 3. Table 4 shows the distribution of analytes grouped according to the calculated sigma values.

The sigma values of Alb, DBIL, Cl, Na, urea, and TG for IQC level 1 and the sigma values of Alb, Cl, and Na for IQC level 2 were <3 on the first analyzer. The sigma values of Cl and Na for IQC level 1, and the sigma values of Alb, DBIL, Cl, and Na for IQC level 2 were <3 on analyzer 2.

The sigma values of ALT, TBIL, P, Glc, HDL-cholesterol, total cholesterol, Ca, CREA, TP, and UA for IQC level 1, and the sigma values of DBIL, TBIL, P, Glc, HDL-cholesterol, total cholesterol,

Table 2. Analyzer 1: Target mean values provided by the manufacturer and the calculated laboratory mean, SD, CV %, bias %, and process sigma level of internal quality controls calculated in our laboratory

Test (Unit)	IQC level	Target mean	TEa % (CLIA'88)	Laboratory mean	Laboratory SD	Laboratory CV %	Laboratory bias %	Sigma level
Albumin (g/L)	QC1	0.03	10	0.031	0.001	2.91	3.13	2.36
	QC2	0.036	10	0.038	0.001	2.81	5.03	1.77
Alkaline phosphatase (IU/L)	QC1	68.5	30	67.93	3.2	4.71	-0.83	6.55
	QC2	183.5	30	193.97	6.13	3.16	5.7	7.69
Alanine aminotransferase (IU/L)	QC1	21.5	20	19.44	1.83	9.43	-9.6	3.14
	QC2	108	20	105.79	2.3	2.17	-2.04	10.16
Aspartate aminotransferase (IU/L)	QC1	37.5	20	38.81	1.06	2.73	3.5	6.04
	QC2	135.5	20	140.67	2.55	1.81	3.81	8.93
Amylase (U/L)	QC1	38	30	38.15	1.06	2.79	0.40	10.61
	QC2	112.5	30	117.43	2.94	2.51	4.38	10.22
Direct bilirubin (µmol/L)	QC1	38.1	20	36.55	3.46	9.48	-3.83	2.52
	QC2	110.5	20	104.37	8.6	8.24	-5.55	3.1
Total bilirubin (µmol/L)	QC1	81.33	20	71.91	4.64	6.46	-11.58	4.89
	QC2	253.71	20	227.77	15.64	6.87	-10.23	4.4
Creatine kinase (IU/L)	QC1	68	30	63.91	1.99	3.11	-6.02	11.57
	QC2	238	30	235.82	5.05	2.14	-0.92	14.45
Iron (µmol/L)	QC1	4.54	20	4.48	0.15	3.34	-1.26	6.36
	QC2	5.6	20	5.48	0.15	2.8	-2.25	7.94
Phosphorus (mg/dL)	QC1	2.25	15	2.34	0.06	2.72	4.03	4.04
	QC2	4.3	14.8	4.45	0.13	2.81	3.45	4.03
Gamma-glutamyl transferase (U/L)	QC1	25	17.9	20.05	1.24	6.19	-19.81	6.1
	QC2	69.5	17.4	65.5	1.82	2.77	-5.76	8.35
Glucose (mmol/L)	QC1	2.78	10	2.75	0.09	3.1	-0.92	3.52
	QC2	7.16	10	7.07	0.16	2.2	-1.31	5.14
HDL-cholesterol (mmol/L)	QC1	1.44	30	1.42	0.08	5.73	-1.42	5.48
	QC2	2.22	30	2.15	0.13	6.27	-3.32	5.31
Total cholesterol (mmol/L)	QC1	5	10	5.04	0.11	2.12	0.85	4.31
	QC2	8.38	10	8.5	0.2	2.36	1.45	3.62
Calcium (mmol/L)	QC1	0.35	11.3	0.35	0.01	2.55	-0.88	4.78
	QC2	0.53	7.4	0.54	0.01	2.25	0.5	3.07
Chloride (mmol/L)	QC1	84.5	5	86.28	1.52	1.76	2.11	1.65
	QC2	99	5	101.55	1.88	1.85	2.57	1.31
Creatinine (µmol/L)	QC1	54.81	15	54.64	1.51	2.77	-0.31	5.53
	QC2	179.45	15	180.09	5.4	3	0.36	4.88
Lactate dehydrogenase (U/L)	QC1	111	20	105.59	3.63	3.44	-4.87	7.24
	QC2	230	20	237.93	8	3.36	3.45	4.93
Lipase (U/L)	QC1	25.5	30	24.72	1.31	5.29	-3.06	6.25
	QC2	70	30	68.81	3.16	4.6	-1.7	6.9
Magnesium (mmol/L)	QC1	0.08	25	0.078	0.002	3.1	-3.03	9.04
	QC2	0.144	25	0.137	0.003	2.56	-4.66	11.6
Potassium (mmol/L)	QC1	2.55	13.9	2.53	0.06	2.27	-0.9	6.52
	QC2	4.25	7	4.25	0.08	1.88	-0.09	3.77
Sodium (mmol/L)	QC1	113	2.7	113.76	1.88	1.65	0.67	1.23
	QC2	146.5	2.9	148.36	2.63	1.77	1.27	0.92
Total protein (g/L)	QC1	46.5	10	47.19	0.75	1.59	1.49	5.36
	QC2	57	10	57.77	0.99	1.72	1.35	5.04
Triglyceride (mmol/L)	QC1	3.83	25	4.1	0.35	8.42	6.96	2.14
	QC2	9.38	25	9.63	0.47	4.93	2.69	4.53
Uric acid (mmol/L)	QC1	0.14	17	0.15	0.004	2.56	6.1	4.26
	QC2	0.31	17	0.32	0.01	2.09	2.62	6.86
Urea (mmol/L)	QC1	0.94	9	0.96	0.04	4.62	1.91	1.53
	QC2	4.77	9	4.78	0.1	2.07	0.13	4.29

CLIA'88: Clinical Laboratory Implementation Amendments 1988; CV: Coefficient of variation; IQC: Internal quality control; TEa: Total allowable error; QC: Quality control; HDL: high-density lipoprotein.

Table 3. Analyzer 2: Target mean values provided by the manufacturer and the calculated laboratory mean, SD, CV %, bias %, and process sigma level of internal quality controls calculated in our laboratory

Test (Unit)	IQC level	Target mean	TEa % (CLIA'88)	Laboratory mean	Laboratory SD	Laboratory CV %	Laboratory bias %	Sigma level
Albumin (g/L)	QC1	0.03	10	0.031	0.001	2.14	3.21	3.18
	QC2	0.036	10	0.038	0.001	1.9	4.33	2.99
Alkaline phosphatase (IU/L)	QC1	68.5	30	67.33	2.28	3.38	-1.7	9.37
	QC2	183.5	30	192.22	5.76	3	4.75	8.43
Alanine aminotransferase (IU/L)	QC1	21.5	20	19.77	0.86	4.36	-8.05	6.43
	QC2	108	20	103.96	1.4	1.35	-3.74	17.64
Aspartate aminotransferase (IU/L)	QC1	37.5	20	37.42	1.03	2.74	-0.21	7.37
	QC2	135.5	20	137	2.43	1.78	1.11	10.64
Amylase (U/L)	QC1	38	30	38.04	0.45	1.2	0.11	25.01
	QC2	112.5	30	115.77	1.42	1.23	2.91	22.04
Direct bilirubin (µmol/L)	QC1	38.01	20	37.16	2.68	7.2	-2.23	3.09
	QC2	110.5	20	105.38	8.72	8.28	-4.63	2.98
Total bilirubin (µmol/L)	QC1	79.56	20	71.83	5.49	7.64	-9.72	3.89
	QC2	253.71	20	231.17	16.92	7.32	-8.89	3.95
Creatine kinase (IU/L)	QC1	68	30	63	1.76	2.79	-7.35	13.4
	QC2	238	30	233.91	5.02	2.14	-1.72	14.79
Iron (µmol/L)	QC1	4.54	20	4.47	0.09	1.94	-1.54	11.1
	QC2	5.6	20	5.46	0.15	2.77	-2.48	8.1
Phosphorus (mg/dL)	QC1	2.25	15	2.34	0.05	2.15	4.07	5.08
	QC2	4.3	14.8	4.47	0.11	2.49	3.84	4.4
Gamma-glutamyl transferase (U/L)	QC1	25	17.9	19.87	0.68	3.44	-20.51	11.16
	QC2	69.5	17.4	65.63	1.57	2.39	-5.56	9.61
Glucose (mmol/L)	QC1	2.78	10	2.74	0.07	2.69	-1.18	4.16
	QC2	7.16	10	7.07	0.15	2.19	-1.28	5.16
HDL-cholesterol (mmol/L)	QC1	1.44	30	1.42	0.09	6.15	-1.94	5.19
	QC2	2.22	30	2.12	0.12	5.64	-4.71	6.15
Total cholesterol (mmol/L)	QC1	5	10	5.07	0.11	2.1	1.56	4.03
	QC2	8.41	10	8.5	0.22	2.53	1.07	3.53
Calcium (mmol/L)	QC1	0.347	11.3	0.345	0.01	1.88	-0.46	6.25
	QC2	0.533	7.4	0.532	0.01	1.97	-0.27	3.9
Chloride (mmol/L)	QC1	84.5	5	86.52	1.62	1.87	2.4	1.4
	QC2	99	5	101.09	1.8	1.78	2.11	1.62
Creatinine (µmol/L)	QC1	54.81	15	53.72	1.79	3.34	-1.99	5.09
	QC2	179.45	15	178.23	5.43	3.04	-0.68	5.15
Lactate dehydrogenase (U/L)	QC1	111	20	104.52	4.71	4.51	-5.84	5.73
	QC2	230	20	235.29	5.46	2.32	2.3	7.63
Lipase (U/L)	QC1	25.5	30	24.28	1.59	6.57	-4.78	5.3
	QC2	70	30	67.5	3.89	5.76	-3.57	5.83
Magnesium (mmol/L)	QC1	0.08	25	0.078	0.002	2.86	-3.02	9.79
	QC2	0.144	25	0.136	0.004	2.95	-5.49	10.34
Potassium (mmol/L)	QC1	2.55	13.9	2.55	0.07	2.73	-0.13	5.14
	QC2	4.25	7	4.27	0.08	1.98	0.35	3.35
Sodium (mmol/L)	QC1	113	2.7	114.81	2.18	1.9	1.6	0.58
	QC2	146.5	2.9	149.27	2.44	1.64	1.89	0.62
Total protein (g/L)	QC1	46.5	10	47.2	0.82	1.73	1.51	4.91
	QC2	57	10	57.88	0.83	1.44	1.54	5.88
Triglyceride (mmol/L)	QC1	3.83	25	3.97	0.09	2.27	3.58	9.44
	QC2	9.38	25	9.63	0.21	2.23	2.72	9.99
Uric acid (mmol/L)	QC1	0.14	17	0.15	0.004	2.81	6.42	3.77
	QC2	0.31	17	0.32	0.01	2.11	1.88	7.18
Urea (mmol/L)	QC1	0.94	9	0.92	0.03	2.78	-2.55	4.15
	QC2	4.77	9	4.69	0.12	2.62	-1.65	4.06

CLIA'88: Clinical Laboratory Implementation Amendments 1988; CV: Coefficient of variation; IQC: Internal quality control; TEa: Total allowable error; QC: Quality control; HDL: high-density lipoprotein.

Table 4. Distribution of analytes grouped according to calculated sigma value

	Analyzer 1		Analyzer 2	
Sigma metrics	**QC1**	**QC2**	**QC1**	**QC2**
Group 1 (<3 sigma)	Albumin Direct bilirubin Chloride Sodium Urea Triglyceride	Albumin Chloride Sodium	Chloride Sodium	Albumin Direct bilirubin Chloride Sodium
Group 2 (3-6 sigma)	ALT Total bilirubin Phosphorus Glucose HDL-cholesterol Total cholesterol Calcium Creatinine Total protein Uric acid	Direct bilirubin Total bilirubin Phosphorus Glucose HDL-cholesterol Total cholesterol Calcium Creatinine Lactate dehydrogenase Potassium Total protein Triglyceride Urea	Albumin Direct bilirubin Total bilirubin Phosphorus Glucose HDL-cholesterol Total cholesterol Creatinine Lactate dehydrogenase Lipase Potassium Total protein Uric acid Urea	Total bilirubin Phosphorus Glucose Total cholesterol Calcium Creatinine Lipase Potassium Total protein Urea
Group 3 (>6 sigma)	Alkaline phosphatase AST Amylase Creatine kinase Iron GGT Lactate dehydrogenase Lipase Magnesium Potassium	Alkaline phosphatase ALT AST Amylase Creatine kinase Iron GGT Lipase Magnesium Uric acid	Alkaline phosphatase ALT AST Amylase Creatine kinase Iron GGT Calcium Magnesium Triglyceride	Alkaline phosphatase ALT AST Amylase Creatine kinase Iron GGT HDL-cholesterol Lactate dehydrogenase Magnesium Triglyceride Uric acid

QC: Quality control; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; HDL: High-density lipoprotein.

Ca, CREA, LD, K, TP, TG, and urea for IQC level 2 were within the acceptable range on analyzer 1 (sigma level: 3-6). For analyzer 2, the sigma values of Alb, DBIL, TBIL, P, Glc, HDL-cholesterol, total cholesterol, CREA, LD, lipase, K, TP, UA, and urea for IQC level 1, and the sigma values of TBIL, P, Glc, total cholesterol, Ca, CREA, lipase, K, TP, and urea for IQC level 2 were within the acceptable range (sigma level: 3-6).

ALP, ALT (except IQC level 1 on analyzer 1), AST, AMY, CK, Fe, lipase (analyzer 1), GGT, LD (except IQC level 2 on analyzer 1 and IQC level 1 on analyzer 2), Mg, TG (analyzer 2), UA (except IQC level 1 on both analyzers), K (IQC level 1 on analyzer 1), Ca (IQC level 1 on analyzer 2), and HDL-cholesterol (IQC level 2 on analyzer 2) had good performance (sigma level: >6).

Discussion

We evaluated 26 biochemical analytes using the Six Sigma methodology on 2 Abbott Architect c16000 analyzers and we compared IQC level sigma values. For both analyzers, the 2 levels of IQC sigma values of Cl, Na, and Alb (except IQC level 1 on analyzer 2) were <3; the 2 levels of IQC sigma values of ALP, AST, AMY, CK, Fe, GGT, Mg, TG (analyzer 2), ALT (except IQC level 1 on analyzer 1) were >6. The 2 levels of IQC sigma values of TBIL, P, Glc, HDL-cholesterol (except IQC level 2 on analyzer 2), total cholesterol, Ca (except IQC level 1 on analyzer 2), CREA, TP, and urea (except IQC level 1 on analyzer 1) were within the acceptable range (sigma level: 3-6). AMY and CK had the best

sigma metrics on both analyzers, while Na had the lowest sigma values on both analyzers. The sigma values of many analytes were consistent for both analyzers.

Many studies have evaluated analytical performance using the Six Sigma method with different analyzers and parameters, and using internal or external quality controls. Medina et al. [12] evaluated 5 years of IQC data of 2 Abbott Architect c8000 chemistry analyzers. The sigma values of DBIL, CK, HDL-cholesterol, TG, and UA were >6 for 1 analyzer while the values of CK, DBIL, HDL-cholesterol, Mg, TG, and UA were >6 for the second analyzer. The electrolytes Ca, Cl, and Na had an average sigma level of <3 on both devices, while K showed better sigma scores.

Mao et al. [1] and Zhou et al. [13] extracted 5 months' worth of IQC data of biochemical parameters using the AU5800 analyzer (Beckman Coulter Inc., Brea, CA, USA). In the study performed by Mao et al. [1], the sigma values of urea and Na were determined to be <3; and AMY, UA, HDL-cholesterol, TBIL, ALT, TG, AST, ALP, and CREA were >6. Zhou et al. [13] reported that the sigma values of BUN, Ca, ALT, and P were <3, and those of ALP, CK, TG, GGT, and TBIL were >6.

Other studies in the literature that used the Six Sigma method have yielded varied results [10, 11, 14-18]. The differences in sigma values of the analytes may be due to differences in the autoanalyzers, the quality control material, the pre/post-analytical conditions, the period of study, or the method used.

The TEa target levels used to evaluate the analytical process influence the calculated sigma values. It has been noted that the different CLIA and RiliBÄK TEa levels affected the results [4, 5]. In our study, the TEa target values were determined according to the CLIA'88 and the low sigma values of Na and Cl seen may have been due to the low TEa target levels used. A very stringent calibration, IQC, and analyzer maintenance have to be followed for parameters with low sigma values. Simple QC rules are adequate for parameters with high sigma values.

Six Sigma-method applications allow a laboratory to calculate their performance using universal criteria and to compare the results with those of other clinical laboratories around the world. Parameters with low performance can be identified using this analysis and performance should be improved with regulatory activities in order to meet the universal quality criteria. The sigma levels of Na, Cl, and Alb in our study indicated that regulatory activities should be conducted for low concentration and electrolyte tests that were studied with the indirect ion selective electrode method. Fluctuations in the electrolyte results may have been due to contamination or deterioration of the reference electrode. We implemented regulatory activities such as changing the reference solution and performing electrode maintenance more frequently to resolve these problems.

This study has some limitations that should be noted. First, the evaluation period was limited to 3 months. Second, because of the short duration, the EQC-Bias % was not evaluated. We believe that EQC-Bias % data calculated over longer periods would provide statistically more accurate results, and our next goal is to evaluate the EQC as well as the IQC over a longer period as a bias indicator.

In conclusion, the Six Sigma method is an effective form of statistical analysis to evaluate analytical process performance with quality control results. In the present study, the identification and measurement steps of the universal application were performed for biochemistry tests on 2 Abbott Architect c16000 analyzers. The next goal is to perform the analysis, improvement, and control steps to further enhance our analytical process performance.

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Research Article

Biosafety risk assessment of a clinical biochemistry laboratory for SARS-CoV2 infection

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Abstract

Objectives: Clinical laboratories are a transfer point for infected patient samples. According to the World Health Organization (WHO) Laboratory Biosafety Guideline, a risk assessment approach is the backbone of laboratory biosafety. In laboratories, risk assessment is recommended at predetermined periods and in the event of new circumstances. On February 12, 2020, the WHO published an interim guidance document, "Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)" and it was highly recommended that all coronavirus 2019 (COVID-19) testing procedures be performed based on a local risk assessment. This study was designed to evaluate the biosafety risk in a biochemistry laboratory where routine testing of patients diagnosed with COVID-19 is performed.

Methods: Risk assessment for tests performed on analyzers and a complete urinalysis was performed using the risk assessment template included in the subsequent WHO interim guidance document, "Laboratory biosafety guidance related to coronavirus disease (COVID-19)."

Results: The overall initial risk for tests performed on analyzers and a complete urinalysis test was determined to be very high. Processes such as pipetting a sample and checking a sample tube by scanning the barcode during tests performed on analyzers were suspended until additional risk control measures could be implemented. The manual microscopic urinalysis process was also discontinued. To reduce the risk, surgical masks, surgical caps, eye protection, and disposable laboratory coats were added to the previously mandated personal protective equipment. After implementing additional risk control measures, the total residual risk of both processes was graded medium.

Conclusion: Since there is as yet no effective treatment for COVID-19, exposure risk is considered severe. Therefore, the probability of exposure is important in determining the level of risk. Measures put in place reduced the total residual risk.

Keywords: Biosafety, COVID-19, laboratory risk assessment, pandemic

Coronavirus 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first reported in Wuhan, China, in December 2019 [1]. It was declared a pandemic by the World Health Organization (WHO) in March 2020 [2]. The first laboratory-confirmed COVID-19 case in Turkey was announced by the Ministry of Health on March 11, 2020 [3].

COVID-19 is an aerosol transmissible disease, and the infectious dose of SARS-CoV-2 remains unknown. Healthy individuals can also be infected by touching contaminated surfaces and then transferring viral particles to the mucous membranes of the eyes, nose, and mouth (indirect contact or fomite transmission) [4].

Viral respiratory infection is confirmed with the detection of viral nucleic acid in tissue samples, which is indicative of active virus replication [5]. Upper respiratory material (nasopharyngeal and oropharyngeal swab or wash in ambulatory patients) or lower respiratory material (sputum and/or endotracheal aspirate or bronchoalveolar lavage) has been defined by the WHO as the minimum sample for a diagnosis of COVID-19. It

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has also been reported that SARS-CoV-2 can be detected in blood and stool [6].

Viral RNA in patient specimens (nasopharyngeal swab, sputum, stool samples, etc.) is detected using the polymerase chain reaction (PCR) method, which targets independent regions of the SARS-CoV-2 nucleocapsid gene [7]. Viral RNA is first purified from the sample and then transcribed into DNA, which is amplified in a PCR reaction to produce easily detectable amounts [4]. Several real-time reverse transcriptase PCR assay protocols have been developed and approved by the US Center for Disease Control and Prevention and are now widely used for the diagnosis of COVID-19 [7].

SARS-CoV-2 RNA can be identified in respiratory samples 1 to 2 days before the onset of symptoms and shedding can take up to 7 to 12 days in moderate cases, and up to 2 weeks in severe cases [8]. The viral load in urine, saliva, and stool samples not currently used in the routine diagnosis of COVID-19 has been shown to be almost equal to or greater than the viral load in nasopharyngeal or oropharyngeal swab samples [9]. The viable virus in stool and serum can be detected for nearly 5 weeks after a respiratory sample has tested negative for SARS-CoV-2 RNA [5, 7]. Therefore, the risk of SARS-CoV-2 transmission via stool and blood samples cannot be excluded.

The minimum biosafety level (BSL) for clinical laboratories is BSL 2. While specimen handling for molecular testing would require BSL 2 facilities or the equivalent, culturing the virus would require a minimum of BSL 3 facilities [6]. Clinical laboratories are an transfer point for infected patient samples. Although laboratory divisions such as hematology, biochemistry, and blood bank departments follow BSL 2 blood-born pathogen standards, these units often lack access to equipment such as a certified biological safety cabinet. Also, although lab workers claim that they follow certain practices and take security measures, daily practice observations do not always support these claims [10]. Consequently, it is recommended that clinical laboratories that work with samples such as blood products and urine containing relatively lower virus concentrations (in comparison with a microbiology laboratory where respiratory samples are processed) should also perform a risk assessment to prevent exposure to aerosols and droplets when processing samples that may contain SARS-CoV-2 [11, 12]. As stated in a WHO Laboratory Biosafety Guideline, a risk assessment approach is the backbone of laboratory biosafety [13]. Risk assessment is the process that collects data about workplace hazards and evaluates the possible results of exposure. Appropriate risk control measures are selected and implemented to reduce the risks identified in the evaluation and mitigate residual risk. Laboratory risk assessment is recommended at predetermined periods and in any instance of new circumstances. On February 12, 2020, the WHO published the interim guidance document, "Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)," which recommended that all procedures related to COVID-19 be performed based on risk assessment [11].

This study is an examination of a biosafety risk assessment performed in a clinical biochemistry laboratory to determine measures to be taken during the COVID-19 pandemic.

Materials and Methods

Risk assessment was performed on analyzers and a complete urinalysis test using the template included in the WHO interim guidance supplement "WHO laboratory biosafety guidance related to coronavirus disease 2019 (COVID-19)" [11].

In our clinical biochemistry laboratory, an ADVIA Centaur XP immunoassay system (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA), a Siemens ADVIA chemistry analyzer (Siemens Healthcare Diagnostic Inc., Deerfield, IL, USA), and a DIRUI H-500 urine analyzer (DIRUI Industrial Co. Ltd., Changchun, Jilin China) are used for immunoassay analysis, chemistry analysis, and urine strip analysis, respectively. The urine strip analyzer is semi-automated; microscopic analysis of urine is performed manually. The assessment steps were based on the standard operating procedure and opportunities for potential exposure and/or release for each process. A brief overview of the evaluations is provided in Figure 1 and Figure 2. An initial risk assessment of the laboratory procedures and the overall risk was estimated for each process. The addition of risk control measures was prioritized according to urgency, feasibility/sustainability, delivery and installation time, and availability. Requirements prescribed by international and national regulations, legislation, guidelines, policies, and strategies on biosafety were also included [13, 14]. In addition, the process examines the applicability, availability, and sustainability of the resources available for risk control as well as the level of residual risk with these risk control measures in place. The residual risk after risk control measures was evaluated for each process to determine if that level of risk would be below the tolerance level and whether work should proceed. Step 2 of the WHO risk assessment examines the consequences of exposure and likelihood of exposure. As there is still no effective treatment for COVID-19, the consequences of any exposure to material that is suspected for COVID-19 is categorized as severe. The maximum acceptable residual risk in this case is a medium risk tolerance level. The overall residual risk of the laboratory processes examined with the benefit of risk control measures was estimated. The highest level of risk for any step of the process before risk assessment was considered the initial overall risk for the whole process. Standard operating procedures for tests performed on analyzers and a complete urinalysis were redefined based on the risk assessment (Fig. 3, 4). Finally, a review was scheduled to be performed every 6 months or upon changes in the laboratory (laboratory staff, analyzer, or process) or knowledge of SARS-CoV-2 (Annex 1, 2).

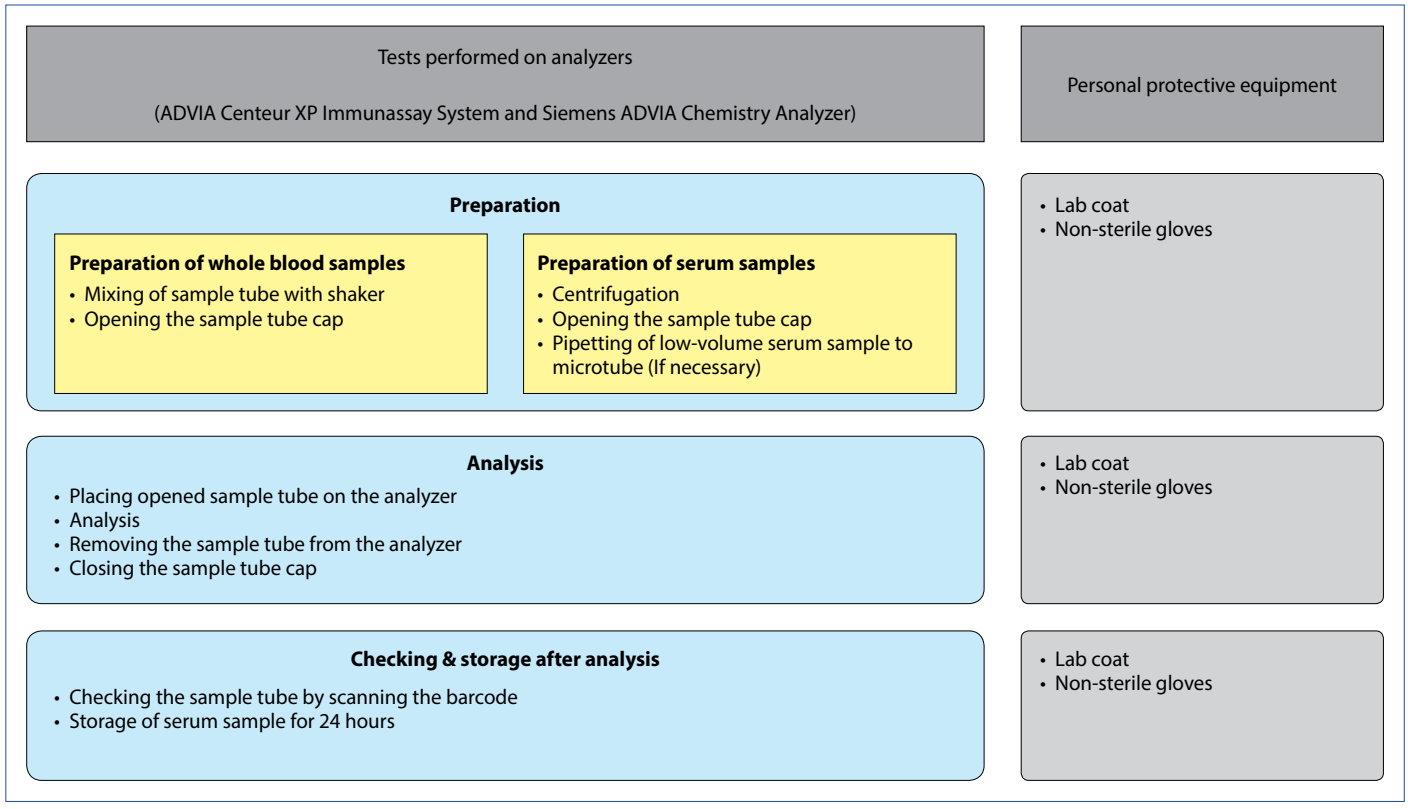

Figure 1. A brief overview of the process for tests performed on analyzers.

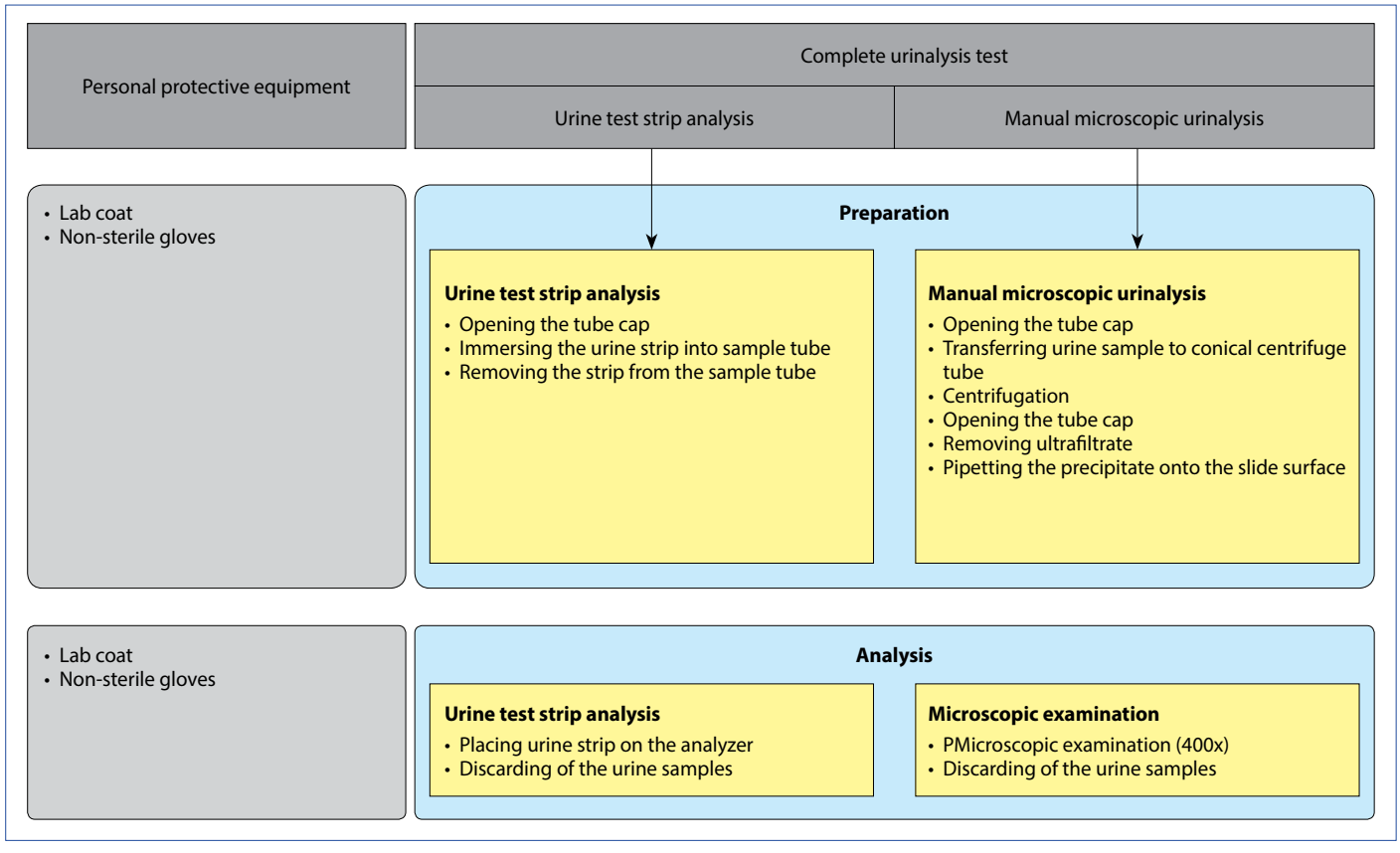

Figure 2. A brief overview of the process for a complete urinalysis test.

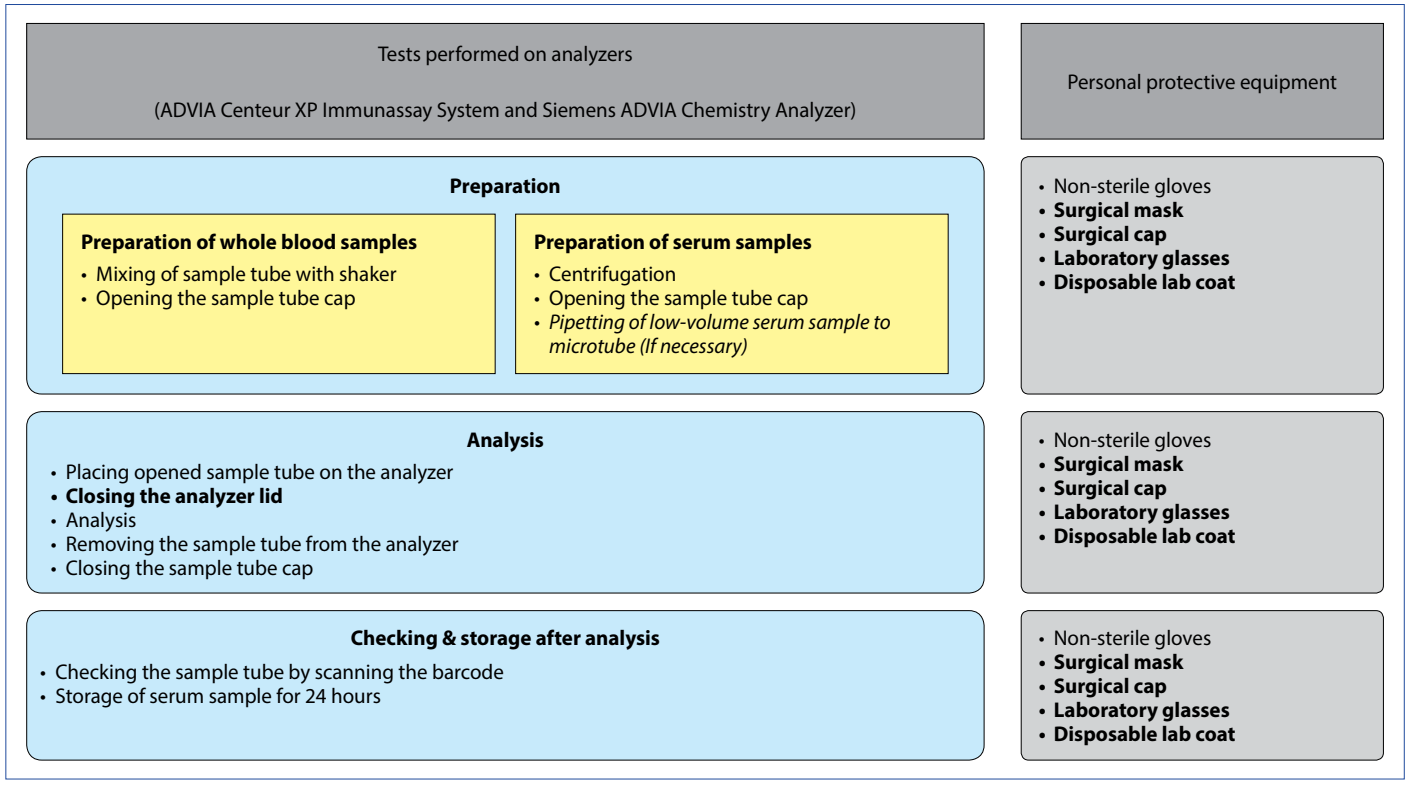

Figure 3. A brief overview after implementation of risk control measures for tests performed on analyzers. Discontinued steps are presented in italic font and new biosafety steps are in bold font.

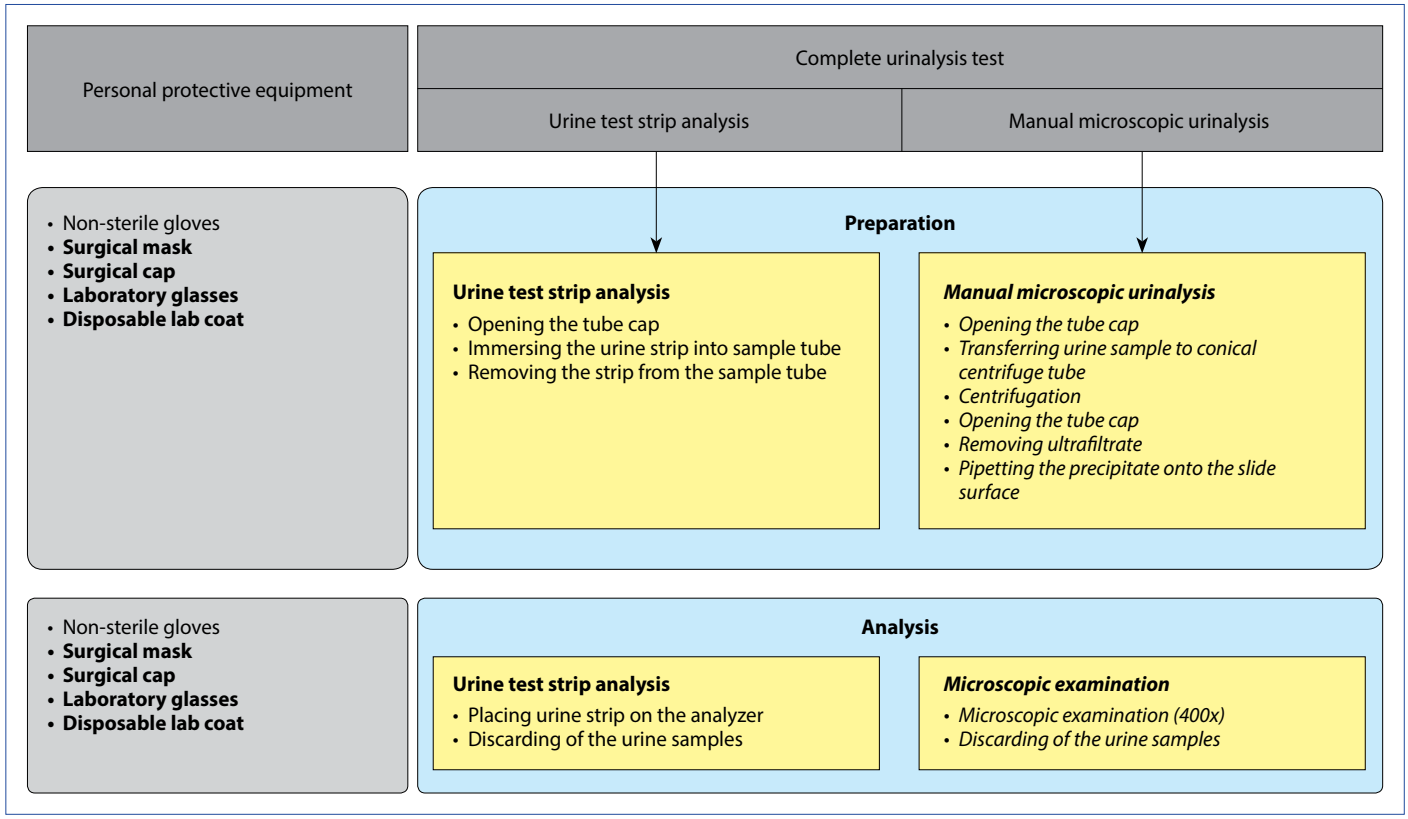

Figure 4. A brief overview after implementation of risk control measures for a complete urinalysis test. Discontinued steps are presented in italic font and new biosafety steps are in bold font.

Results

Examination of tests performed on analyzers revealed that the opening or closing of a sample tube cap, pipetting of specimens, placing opened sample tubes on the analyzer, analysis procedures, removing the sample tube from the analyzer, and checking sample tubes by scanning barcodes were steps with the risk of generating aerosols that could include SARS-CoV-2.

The initial risk classification for pipetting a specimen was very high, while it was graded high for the analysis step, and medium for opening or closing the sample tube cap, placing opened samples tube on the analyzer, removing the sample tube from the analyzer, and checking the sample tube by scanning the barcode (Table 1). The overall initial risk for the analyzer testing process was determined to be very high. It was decided that additional risk control measures were necessary. The steps of pipetting the specimen and checking the sample tube by scanning the barcode were discontinued (Table 2). To reduce the risk of contact, surgical masks, surgical caps, eye protection, and disposable laboratory coats were added to the previously used personal protective equipment.

Table 1. The initial risk assessment for tests performed on analyzers

		Likelihood of exposure/release		
		Unlikely	**Possible**	**Likely**
Consequence of exposure/release	Severe	Medium	High	Very high
	Moderate	Low	Medium	High
	Negligible	Very low	Low	Medium
Laboratory activity/procedure		Initial risk (very low, low, medium, high, very high)	Is the initial risk above the tolerance level? (yes/no)	Priority (high/medium/low)
Opening or closing the sample tube cap		Severe x Unlikely=Medium	No	Low
Pipetting of specimen		Severe x Likely=Very high	Yes	High
Placing opened sample tube on the analyzer		Severe x Unlikely=Medium	No	Low
Analysis		Severe x Possible=High	Yes	High
Removing the sample tube from the analyzer		Severe x Unlikely=Medium	No	Low
Checking the sample tube by scanning the barcode		Severe x Unlikely=Medium	No	Low
Select the overall initial risk		☐ Very low ☐ Low	☐ Medium ☐ High	☑ Very high
Should work proceed without additional risk control measures?		☐ Yes	☑ No	

Table 2. The risk assessment for tests performed on analyzers after the adoption of control measures

Laboratory activity/procedure	**Selected risk control measure(s)**	**Residual risk (very low, low, medium, high, very high)**	**Is the residual risk above the tolerance level? (yes/no)**	**Are risk control measures available, effective and sustainable? (yes/no)**
Opening or closing the sample tube cap	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	Severe x Unlikely=Medium	No	Yes
Pipetting of specimen	Discontinued	-	No	-
Placing opened sample tube on the analyzer	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	Severe x Unlikely=Medium	No	Yes
Analysis	Analyzer lid closed; surgical mask, surgical cap, laboratory glasses, disposable lab coat	Severe x Unlikely=Medium	No	Yes
Removing the sample tube from the analyzer	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	Severe x Unlikely=Medium	No	Yes
Checking the sample tube by scanning the barcode	Discontinued	-	No	-
Overall residual risk	☐ Very low ☐ Low	☑ Medium	☐ High	☐ Very high

Table 3. The initial risk assessment for a complete urinalysis test

		Likelihood of exposure/release				
		Unlikely		**Possible**		**Likely**
Consequence of exposure/ release	Severe	Medium		High		Very high
	Moderate	Low		Medium		High
	Negligible	Very low		Low		Medium
Laboratory activity/procedure		Initial risk (very low, low, medium, high, very high)		Is the initial risk above the tolerance level? (yes/no)		Priority (high/ medium/low)
Immersing the urine strip into sample tube, removing the strip from the sample tube		Severe x Unlikely=Medium		No		Low
Transferring urine sample to conical centrifuge tube (pipetting)		Severe x Likely=Very high		Yes		High
Opening the tube cap		Severe x Unlikely=Medium		No		Low
Pipetting		Severe x Likely=Very high		Yes		High
Removing ultrafiltrate		Severe x Likely=Very high		Yes		High
Pipetting the precipitate onto the slide surface		Severe x Likely=Very high		Yes		High
Microscopic examination of stained slides		Severe x Possible=High		Yes		High
Discarding of the urine samples		Severe x Unlikely=Medium		No		Low
Select the overall initial risk		☐ Very low	☐ Low	☐ Medium	☐ High	☑ Very high
Should work proceed without additional risk control measures?		☐ Yes	☑ No			

As a result, the general residual risk for tests performed on an analyzer was reduced to medium (Table 2). A summary of the standard operating procedures following the addition of risk control measures is shown in Figure 3.

The risk of generating aerosols containing SARS-CoV-2 was identified in the urine test strip analysis process steps of opening the tube cap, immersing the urine strip into a sample tube, removing the strip from a sample tube, and disposal of the sample. During the manual microscopic urinalysis, transferring the urine sample to a conical centrifuge tube (pipetting), opening the tube cap, removing ultrafiltrate, pipetting the precipitate onto the slide surface, microscopic examination of stained slides, and discarding the sample were determined to be the steps with the most risk.

The initial risk rating was very high for the transfer and removal of ultrafiltrate, while microscopic examination of stained slides was considered a high risk, and opening the tube cap, immersing the urine strip into a sample tube, removing the strip from the sample tube, and disposal were classified as a medium risk (Table 3). The overall initial risk for a complete urinalysis test was very high. Additional risk control measures were considered necessary (Table 3). The manual microscopic urinalysis process was discontinued. Once additional risk control measures were implemented, the total residual risk of the process for a complete urinalysis was evaluated as medium level (Table 4). A summary of the revised standard operating procedures is shown in Figure 4.

Discussion

Since there is currently no effective treatment for COVID-19, determining the probability of exposure is important. In our risk assessment of analyzer testing, the greatest possibility of aerosolization was in the steps of opening and closing the tube cap, pipetting for transfer, placing the sample tube on the device, analysis (pipetting), and barcode scanning of the tube. The overall initial risk of the process was determined to be very high. To reduce the risk, surgical masks, surgical caps, eye protection, and disposable laboratory coats were added to the personal protective equipment already in use. Measures were implemented to reduce contact between the laboratory workers and the patient samples. The pipetting process (transfer of patient samples with low sample volume to a microtube) and barcode scanning of the sample tubes were eliminated and insufficient samples were rejected. It was also decided that the procedures for the final check of the patient samples would be performed using only the laboratory information system. It was observed that the lid of the sample pipetting area of the analyzer remained open during daily practice. As a result of closing the lid of the analyzer, the risk of the analysis process decreased from high to medium due to reduced exposure to aerosolization that may occur by pipetting inside the instrument. The final residual risk rating after applying risk control measures related to analyzer testing was medium.

In our laboratory, urine microscopic examination and strip analysis are performed manually. These processes were evalu-

Table 4. The risk assessment for a complete urinalysis test after the adoption of control measures

Laboratory activity/ procedure	Selected risk control measure(s)	Residual risk (very low, low, medium, high, very high)	Is the residual risk above the tolerance level? (yes/no)	Are risk control measures available, effective and sustainable? (yes/no)
Immersing the urine strip into sample tube, removing the strip from the sample tube	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	Severe x Unlikely=Medium	No	Yes
Transferring urine sample to conical centrifuge tube (pipetting)	Discontinued	-	No	-
Opening the tube cap	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	Severe x Unlikely=Medium	No	Yes
Pipetting	Discontinued	-	No	-
Removing ultrafiltrate	Discontinued	-	No	-
Pipetting the precipitate onto the slide surface	Discontinued	-	No	-
Microscopic examination of stained slides	Discontinued	-	No	-
Discarding of the urine samples	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	Severe x Unlikely=Medium	No	Yes
Overall residual risk	☐ Very low ☐ Low	☑ Medium	☐ High	☐ Very high

ated separately in the risk assessment of the complete urinalysis test process. In the urine test strip analysis process, opening the tube cap, immersing and removing the urine strip from a sample tube, and disposal were determined to be steps that could generated aerosols. Transferring the urine sample into a conical centrifuge tube, opening the tube cap, removing the ultra-filtrate, pipetting the precipitate onto the slide surface, and microscopic examination procedures were the steps that could lead to aerosolization of SARS-CoV-2 in the manual microscopic analysis. Pipetting was found to be a high risk step due to high exposure probability. The remaining steps were evaluated as medium risk. The overall initial risk of a complete urinalysis test was considered very high.

Human coronaviruses other than SARS-CoV and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) are classified in Risk Group 2 according to Appendix B of the US National Institute of Health Guideline for Research Involving Recombinant or Synthetic Nucleic Acid Molecules [15]. SARS-CoV and MERS-CoV cause severe lower respiratory tract disease and are classified in Risk Group 3.

At the time of writing, the SARS-CoV-2 risk group has not yet been identified. The US Centers for Disease Control and Prevention guideline recommends that some standard practices used in the laboratory during the manipulation of potentially infected samples be performed in a certified class 2 biosafety cabinet (BSC), and at least within a BSL 2 laboratory [12].

Our laboratory includes microbiology and biochemistry departments. There is 1 BSC 2A cabinet in the microbiology department and it is in constant use by that staff. It is also not possible to transfer patient samples to a different unit for pipetting due to the physical conditions of the laboratory. Therefore, steps that could result in aerosol formation, such as pipetting performed by the biochemistry department, cannot be performed in the BSC 2A cabinet.

Pipetting is used in many steps of the manual microscopic examination process. In addition, accidents such as leakage between the coverslip or cracking of the coverslip are not uncommon during microscopic examination in daily practice. In such cases, there is typically 15 to 20 cm between the urine sample and the nose of the examiner. Transmission of SARS-CoV-2 through respiratory droplets and direct contact has been widely acknowledged, but viral shedding in urine has also been reported, and infection through infected urine remains a possibility [16].

The urine strip analysis panel performed in our laboratory includes leukocyte esterase and hemoglobin analyses. In a previous study, our performance of urine strip analysis was classified as good [17]. Therefore, the manual microscopic evaluation analysis process of a complete urinalysis test was suspended temporarily because the risk level could not be reduced.

The unknown properties of the pathogen of the current pandemic present several difficulties and concerns. Laboratories should be aware that any sample may contain high-risk pathogens and therefore a safe environment for laboratory staff is essential. This requires the support of not only laboratory experts, but the administrative and working staff as well [13, 18].

Communication is a vital point in risk assessment and control. The measures to be taken will only be as effective as the employees' awareness of risk. The technicians working in our biochemistry laboratory had no experience working in a laboratory with high-risk pathogens. Therefore, their awareness of the use of personal protective equipment was not at a sufficient level. After completing the risk assessment, detailed in-service training was provided for all laboratory staff, including the revised standard operating procedures.

Conclusion

The risk assessment process has established steps; however, it should not be forgotten that it is subjective and the results of one process are not a recipe that can be used for each process or each laboratory. This study examined a risk assessment applied to routine laboratory processes during the COVID-19 pandemic. The tools used and the results may be useful to other clinical laboratories as a means to transfer recommended risk assessment guidelines to daily practice.

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Ethics Committee Approval: Not applicable.

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Peer-review: Externally peer-reviewed.

Authorship Contributions: Concept – N.Z., O.Z.; Design – N.Z., O.Z.; Supervision – N.Z., O.Z.; Funding – None; Materials – N.Z.; Data collection &/or processing – N.Z.; Analysis and/or interpretation – N.Z., O.Z.; Literature search – N.Z., O.Z.; Writing – N.Z., O.Z.; Critical review – O.Z.

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Annex 1. World Health Organization risk assessment tool used for tests performed on analyzers.

Risk Assessment Template

Although a qualitative approach to combining likelihood and severity parameters in a risk matrix is provided as a risk evaluation method here, it is important to note that quantitative (for example, simple numerical scoring schemes to complex mathematical models) and hybrid (semi-quantitative) methods can also be used for risk evaluation. Laboratories should use a risk evaluation/assessment method that best meets their unique needs, without excluding the possibility of developing customized evaluation approaches, scoring methods and definitions of the parameters.

While this template was primarily developed for biosafety risk assessment, it can also be used for general safety risk assessment of laboratory activities, especially when the biosafety and general safety risks are interlinked, for example, sample collection and transport, where appropriate and applicable.

Institution/Facility name	Kemalpasa State Hospital
Laboratory name	Clinical Biochemistry Laboratory
Laboratory manager/Supervisor	Dr. Nergiz Zorbozan/Specialist of Medical Biochemistry
Project titles/Relevant standard operating procedures (SOPs)	Tests Performed on auto analyzers
Date	March 11, 2020

STEP 1. Gather information (hazard identification)

Instructions: Provide a brief overview of the laboratory work and summarize the laboratory activities to be conducted that are included in the scope of this risk assessment.

Describe the biological agents and other potential hazards (for example, transmission, infectious dose, treatment/preventive measures, pathogenicity).	SARS-CoV-2; • Aerosol transmission, infectious dose unknown, treatment NA, vaccine NA
Describe the laboratory procedures to be used (for example, culturing, centrifugation, work with sharps, waste handling, frequency of performing the laboratory activity).	• Centrifugation • Opening the sample tube cap • Pipetting of low-volume serum sample to microtube (if necessary) • Placing opened sample tube on the analyzer • Analysis • Removing the sample tube from the analyzer • Closing the sample tube cap • Checking the sample tube by scanning the barcode
Describe the types of equipment to be used (personal protective equipment (PPE), centrifuges, autoclaves, biological safety cabinets (BSCs)).	Automatic pipette Centrifuge Autoanalyzer Disposable gloves Lab coat
Describe the type and condition of the facility where work is conducted.	Routine biochemistry laboratory of a secondary healthcare center where COVID-19 cases are also treated. Tests are performed daily.
Describe relevant human factors (for example, competency, training, experience and attitude of personnel).	Patient samples are processed by 8 technicians. Three technicians have been working in the same laboratory for 2 years and the remaining staff for at least 5 years.
Describe any other factors that may affect laboratory operations (for example, legal, cultural, socioeconomic).	Technicians have no experience working with high-risk pathogens with aerosol transmission. Therefore, their awareness of the use of personal protective equipment is inadequate. In general, except for extraordinary situations, the use of personal protective equipment is not given enough attention.

STEP 2. Evaluate the risks

Instructions: Describe how exposure and/or release could occur.

What potential situations are there in which exposure or release could occur?	Aerosols, including SARS-CoV-2, can be generated during the opening and closing of a sample tube cap, pipetting of specimen, placing opened sample tube on the analyzer, analysis (due to pipetting), and checking the sample tube by scanning the barcode.
What is the likelihood of an exposure/release occurring? • Unlikely: not very possible to occur in the near future • Possible: feasible to occur in the near future • Likely: very possible to occur in the near future	Likely
What is the severity of the consequences of an exposure/release (negligible, moderate, severe)?	The consequences of an exposure are severe because to date there is no effective therapy or vaccine for COVID-19.

Instructions: Evaluate the risk and prioritize the implementation of risk control measures. Circle the initial (inherent) risk of the laboratory activities before additional risk control measures have been put in place.

Note:

• When assigning priority, other factors may need to be considered, for example, urgency, feasibility/sustainability of risk control measures, delivery and installation time and training availability.

• To estimate the overall risk, take into consideration the risk ratings for the individual laboratory activities/procedures, separately or collectively as appropriate for the laboratory.

		Likelihood of exposure/release		
		Unlikely	**Possible**	**Likely**
Consequence of exposure/release	Severe	Medium	High	Very high
	Moderate	Low	Medium	High
	Negligible	Very low	Low	Medium
Laboratory activity/procedure		Initial risk (very low, low, medium, high, very high)	Is the initial risk above the tolerance level? (yes/no)	Priority (high/medium /low)
Opening or closing the sample tube cap		S x U=Medium	No	Low
Pipetting of specimen		S x L=Very high	Yes	High
Placing opened sample tube on the analyzer		S x U=Medium	No	Low
Analysis		S x P=High	Yes	High
Removing the sample tube from the analyzer		S x U=Medium	No	Low
Checking the sample tube by scanning the barcode		S x U=Medium	No	Low
Select the overall initial risk.		☐ Very low ☐ Low	☐ Medium ☐ High	☑ Very high
Should work proceed without additional risk control measures?		☐ Yes ☑ No		

STEP 3. Develop a risk control strategy	
Instructions: List any requirements that have been prescribed by international and national regulations, legislation, guidelines, policies and strategies on biosafety and biosecurity.	
Describe the measures required by national legislation or regulations (if any).	-
Describe the measures advised by guidelines, policies and strategies (if any).	It is advised that laboratory procedures that can generate aerosols are to be done in a biological safety cabinet in the World Health Organization document, "Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)."

Instructions: Describe the resources available for risk control and consider their applicability, availability and sustainability in the local context including management support.	
Are resources sufficient to secure and maintain potential risk control measures?	There is no class IIA biological safety cabinet available in our laboratory for relocation of aerosol-generating steps.
What factors exist that may limit or restrict any of the risk control measures?	-
Will work be able to proceed without any of the risk control measures; are there alternatives?	Work cannot proceed without risk control measures.

STEP 4. Select and implement risk control measures

Instructions: Describe where and when risk control measures are needed, the level of residual (remaining) risk when these risk control measures are in place, and an assessment of the availability, effectiveness and sustainability of the risk control measures.

Laboratory activity/procedure	Selected risk control measure(s)	Residual risk (very low, low, medium, high, very high)	Is the residual risk above the tolerance level? (yes/no)	Are risk control measures available, effective and sustainable? (yes/no)
Opening or closing the sample tube cap	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	S x U=Medium	No	Yes
Pipetting of specimen	Discontinued	-	No	-
Placing opened sample tube on the analyzer	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	S x U=Medium	No	Yes
Analysis	Analyzer lid closed. Surgical mask, surgical cap, laboratory glasses, disposable lab coat	S x U=Medium	No	Yes
Removing the sample tube from the analyzer	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	S x U=Medium	No	Yes
Checking the sample tube by scanning the barcode	Discontinued	-	No	-

Instructions: Evaluate the residual risk that remains after risk control measures have been selected to determine if that level of risk is now below the tolerance level and whether work should proceed.

Circle the residual risk of the laboratory activities after risk control measures are in place.

		Likelihood of exposure/release		
		Unlikely	**Possible**	**Likely**
Consequence of exposure/release	Severe	Medium	High	Very high
	Moderate	Low	Medium	High
	Negligible	Very low	Low	Medium
Overall residual risk:		☐ Very low ☐ Low	☑ Medium ☐ High	☐ Very high

If the residual risk is still above the risk tolerance level, further action is necessary such as additional risk control measures, based on the initial risk evaluated in STEP 2, redefining the scope of work such that it falls below the risk tolerance level with existing risk control measures in place or identifying an alternative laboratory with appropriate risk control strategies already in place that is capable of conducting the work as planned.

Should work proceed with selected risk control measures?	☑ Yes ☐ No
Approved by (Name and title)	Dr. Nergiz Zorbozan/Specialist of Medical Biochemistry
Approved by (Signature)	
Date	March 11, 2020

Instructions: Describe how to communicate risks and risk mitigation strategies to personnel. Provide a mechanism of communication within the laboratory. Describe the process and timeline for ensuring that all identified risk control measures are purchased, have associated SOPs and training has been completed before starting the laboratory work.	
Communication of the hazards, risks and risk control measures	Training for laboratory personnel on risks and risk-mitigation strategies was arranged.
Purchase (and budgeting) of risk control measures	-
Operational and maintenance procedures	The transfer of low-volume serum samples to microtube was suspended.
Training of personnel	Training on the revised operational procedures was planned.

STEP 5. Review risks and risk control measures

Instructions: Establish a periodic review cycle to identify: changes in laboratory activities, biological agents, personnel, equipment or facilities; changes in knowledge of biological agents or processes; and lessons learnt from audits/inspections, personnel feedback, incidents and/or near misses.	
Frequency of the review	Every 6 months or upon significant change
Person to conduct the review	Dr. Nergiz Zorbozan/Specialist of Medical Biochemistry
Describe updates/changes	Risks will be reviewed when the COVID-19 conditions in our region have changed.
Personnel/procedures to implement the changes	Lab. Tech. Hakan Acay
Reviewed by (Name and title)	
Reviewed by (Signature)	
Date	

Annex 2. World Health Organization risk assessment tool used for urinalysis.

Risk Assessment Template

Although a qualitative approach to combining likelihood and severity parameters in a risk matrix is provided as a risk evaluation method here, it is important to note that quantitative (for example, simple numerical scoring schemes to complex mathematical models) and hybrid (semi-quantitative) methods can also be used for risk evaluation. Laboratories should use a risk evaluation/assessment method that best meets their unique needs, without excluding the possibility of developing customized evaluation approaches, scoring methods and definitions of the parameters.

While this template was primarily developed for biosafety risk assessment, it can also be used for general safety risk assessment of laboratory activities, especially when the biosafety and general safety risks are interlinked, for example, sample collection and transport, where appropriate and applicable.

Institution/Facility name	Kemalpasa State Hospital
Laboratory name	Clinical Biochemistry Laboratory
Laboratory manager/Supervisor	Dr. Nergiz Zorbozan/Specialist of Medical Biochemistry
Project titles/Relevant standard operating procedures (SOPs)	Urine test strip analysis
	Manual microscopic urinalysis
Date	March 11, 2020

STEP 1. Gather information (hazard identification)

Instructions: Provide a brief overview of the laboratory work and summarize the laboratory activities to be conducted that are included in the scope of this risk assessment.

Describe the biological agents and other potential hazards (for example, transmission, infectious dose, treatment/preventive measures, pathogenicity).	SARS-CoV-2; • Transmission by aerosols, infectious dose unknown, treatment NA, vaccine NA
Describe the laboratory procedures to be used (for example, culturing, centrifugation, work with sharps, waste handling, frequency of performing the laboratory activity).	**Urine test strip analysis** • Opening the tube cap • Immersing the urine strip into the sample tube • Removing the strip from the sample tube **Manual microscopic urinalysis** • Opening the tube cap • Transferring urine sample to conical centrifuge tube • Centrifugation • Removing ultrafiltrate • Pipetting the precipitate onto the slide surface
Describe the types of equipment to be used (personal protective equipment (PPE), centrifuges, autoclaves, biological safety cabinets (BSCs)).	Automatic pipette Centrifuge Disposable gloves Lab coat Microscope Strip analyzer
Describe the type and condition of the facility where work is conducted.	Routine clinical biochemistry laboratory of a secondary healthcare center where COVID-19 cases are also treated. Tests are performed daily.
Describe relevant human factors (for example, competency, training, experience and attitude of personnel).	Patient samples are processed by 8 technicians. Three technicians have been working in the laboratory for 2 years and the remainder for at least 5 years.
Describe any other factors that may affect laboratory operations (for example, legal, cultural, socioeconomic).	Technicians have no experience working with high-risk, aerosol-transmissible pathogens. Therefore, their awareness of the use of personal protective equipment is inadequate. In general, except for extraordinary situations, the use of personal protective equipment is not given enough attention.

STEP 2. Evaluate the risks

Instructions: Describe how exposure and/or release could occur.

What potential situations are there in which exposure or release could occur?	Aerosols, including SARS-CoV-2, can be generated during opening the tube cap, immersing the urine strip into the sample tube, removing the strip from the sample tube, transferring the urine sample to the conical centrifuge tube, opening the tube cap, removing the ultrafiltrate, pipetting the precipitate onto the slide surface, microscopic examination, discarding the urine samples.
What is the likelihood of an exposure/release occurring? • Unlikely: not very possible to occur in the near future • Possible: feasible to occur in the near future • Likely: very possible to occur in the near future	Likely
What is the severity of the consequences of an exposure/release (negligible, moderate, severe)?	The consequences of an exposure are severe because there is currently no effective therapy or vaccine for COVID-19.

Instructions: Evaluate the risk and prioritize the implementation of risk control measures. Circle the initial (inherent) risk of the laboratory activities before additional risk control measures have been put in place.

Note:

• When assigning priority, other factors may need to be considered, for example, urgency, feasibility/sustainability of risk control measures, delivery and installation time and training availability.

• To estimate the overall risk, take into consideration the risk ratings for the individual laboratory activities/procedures, separately or collectively as appropriate for the laboratory.

		Likelihood of exposure/release		
		Unlikely	**Possible**	**Likely**
Consequence of exposure/release	Severe	Medium	High	Very high
	Moderate	Low	Medium	High
	Negligible	Very low	Low	Medium
Laboratory activity/procedure		Initial risk (very low, low, medium, high, very high)	Is the initial risk above the tolerance level? (yes/no)	Priority (high/medium /low)
Immersing the urine strip into the sample tube, removing the strip from the sample tube		S x U=Medium	No	Low
Transferring the urine sample to the conical centrifuge tube (pipetting)		S x L=Very high	Yes	High
Opening the tube cap		S x U=Medium	No	Low
Pipetting		S x L=Very high	Yes	High
Removing ultrafiltrate		S x L=Very high	Yes	High
Pipetting the precipitate onto the slide surface		S x L=Very high	Yes	High
Microscopic examination of stained slides		S x P=High	Yes	High
Discarding the urine samples		S x U=Medium	No	Low
Select the overall initial risk.		☐ Very low ☐ Low	☐ Medium ☐ High	☑ Very high
Should work proceed without additional risk control measures?		☐ Yes ☑ No		

STEP 3. Develop a risk control strategy

Instructions: List any requirements that have been prescribed by international and national regulations, legislation, guidelines, policies and strategies on biosafety and biosecurity.

Describe the measures required by national legislation or regulations (if any).	-
Describe the measures advised by guidelines, policies and strategies (if any).	The World Health Organization document, "Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)" advises that aerosol-generating laboratory procedures are to be performed in a biological safety cabinet.

Instructions: Describe the resources available for risk control and consider their applicability, availability and sustainability in the local context including management support.

Are resources sufficient to secure and maintain potential risk control measures?	We have no class IIA biological safety cabinet available in our biochemistry laboratory for relocation of aerosol-generating steps.
What factors exist that may limit or restrict any of the risk control measures?	-
Will work be able to proceed without any of the risk control measures; are there alternatives?	Work cannot proceed without risk control measures.

STEP 4. Select and implement risk control measures

Instructions: Describe where and when risk control measures are needed, the level of residual (remaining) risk when these risk control measures are in place, and an assessment of the availability, effectiveness and sustainability of the risk control measures.

Laboratory activity/procedure	**Selected risk control measure(s)**	**Residual risk (very low, low, medium, high, very high)**	**Is the residual risk above the tolerance level? (yes/no)**	**Are risk control measures available, effective and sustainable? (yes/no)**
Immersing the urine strip into sample tube, removing the strip from the sample tube	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	S x U=Medium	No	Yes
Transferring the urine sample to a conical centrifuge tube (pipetting)	Discontinued	-	No	-
Opening the tube cap	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	S x U=Medium	No	Yes
Pipetting	Discontinued	-	No	-
Removing ultrafiltrate	Discontinued	-	No	-
Pipetting the precipitate onto the slide surface	Discontinued	-	No	-
Microscopic examination of stained slides	Discontinued	-	No	-
Discarding the urine samples	Surgical mask, surgical cap, laboratory glasses, disposable lab coat	S x U=Medium	No	Yes

Instructions: Evaluate the residual risk that remains after risk control measures have been selected to determine if that level of risk is now below the tolerance level and whether work should proceed.

Circle the residual risk of the laboratory activities after risk control measures are in place.

		Likelihood of exposure/release				
		Unlikely		**Possible**		**Likely**
Consequence of exposure/release	Severe	Medium		High		Very high
	Moderate	Low		Medium		High
	Negligible	Very low		Low		Medium
Overall residual risk:		☐ Very low	☐ Low	☑ Medium	☐ High	☐ Very high

If the residual risk is still above the risk tolerance level, further action is necessary such as additional risk control measures, based on the initial risk evaluated in STEP 2, redefining the scope of work such that it falls below the risk tolerance level with existing risk control measures in place or identifying an alternative laboratory with appropriate risk control strategies already in place that is capable of conducting the work as planned.

Should work proceed with selected risk control measures?	☑ Yes ☐ No
Approved by (Name and title)	Dr. Nergiz Zorbozan/Specialist of Medical Biochemistry
Approved by (Signature)	
Date	March 11, 2020

Instructions: Describe how to communicate risks and risk mitigation strategies to personnel. Provide a mechanism of communication within the laboratory. Describe the process and timeline for ensuring that all identified risk control measures are purchased, have associated SOPs and training has been completed before starting the laboratory work.

Communication of the hazards, risks and risk control measures	Training for laboratory personnel on risks and risk-mitigation strategies was planned.
Purchase (and budgeting) of risk control measures	-
Operational and maintenance procedures	Fresh microscopic examination was discontinued.
Training of personnel	Training for the laboratory technicians on the revised operational procedures was arranged.

STEP 5. Review risks and risk control measures

Instructions: Establish a periodic review cycle to identify: changes in laboratory activities, biological agents, personnel, equipment or facilities; changes in knowledge of biological agents or processes; and lessons learnt from audits/inspections, personnel feedback, incidents and/or near misses.

Frequency of the review	Every 6 months or upon significant change.
Person to conduct the review	Dr. Nergiz Zorbozan/Specialist of Medical Biochemistry
Describe updates/changes	Risks will be reviewed when the COVID-19 conditions in our region have changed.
Personnel/procedures to implement the changes	Lab. Tech. Hakan Acay
Reviewed by (Name and title)	
Reviewed by (Signature)	
Date	

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Research Article

The effects of preconditioning with IFN-γ, IL-4, and IL-10 on costimulatory ligand expressions of mesenchymal stem cells

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Abstract

Objectives: Mesenchymal stem cells (MSCs) are strong immunomodulatory cells, and co-stimulation may play an important role in increasing the effects of MSCs on adaptive immune cells. Preconditioning may add to the effectiveness of MSCs. The aim of this study was to investigate alterations in the costimulatory ligand expressions of MSCs preconditioned with inflammatory cytokines.

Methods: MSCs were preconditioned with interferon gamma (IFN-γ), interleukin (IL) 4 (IL-4), and IL-10, and changes in CD80, CD86, CD137L, CD252, CD274, CD275, and human leukocyte antigen (HLA) class I and II expressions were analyzed using flow cytometry and quantitative polymerase chain reaction methods. Human acute monocytic leukemia cell line (THP-1) macrophages preconditioned under the same conditions served as a control for comparison.

Results: The frequencies of CD80 ($p=0.0003$), CD86 ($p<0.0001$), CD137L ($p<0.0001$), CD252 ($p=0.0003$), CD274 ($p=0.0077$), CD275 ($p<0.0001$), and HLA-II ($p<0.0001$) -positive MSCs was significantly lower than that of the THP-1 macrophages with either method, but there was no significant difference in the HLA-I ($p=0.1506$) cells. Comparison of the expression of the costimulatory ligands revealed that the expression of MSCs was significantly lower than that of THP-1 cells, and was not affected by cytokine stimuli.

Conclusion: The study data indicated that although MSCs are strong immunomodulatory cells, the costimulatory ligand expression required for an effective antigen presentation was extremely low compared with that of professional antigen presenting cells. In addition, preconditioning with IFN-γ, IL-4, and IL-10 failed to increase the expression of important costimulatory ligands, such as CD80 and CD86, in MSCs. The stability of costimulatory ligand expression suggests that MSCs may be an effective source for HLA-I-mediated peripheral tolerance.

Keywords: Costimulatory ligands, interferon gamma, interleukin 4, interleukin 10, mesenchymal stem cells, preconditioning

Mesenchymal stem cells (MSCs) are powerful immunomodulatory cells. Molecules such as prostaglandin E2 (PGE2), indoleamine-pyrrole 2,3-dioxygenase (IDO), transforming growth factor-beta (TGF-β), interleukin (IL) 10, and hepatocyte growth factor (HGF) play an important role in the formation of these effects [1–3]. Molecules on the cell surface of MSCs are another mechanism that suppresses immune cells through cell contact. Strong immunosuppressive molecules, such as programmed death-ligand 1 (PD-L1/CD274), human leukocyte antigen (HLA) G, and B7-Homolog 3 (CD276) are

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highly expressed by MSCs [4–6]. Therefore, they are used experimentally in the treatment of many autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and Crohn's disease. However, most studies have reported that the clinical effects of MSCs are variable and disappear in 3 to 9 months [7]. Several approaches have been used to improve the effectiveness of MSCs. The most common is preconditioning with a specific stimulus, such as hypoxia, drugs, or biological factors [8, 9]. Interferon gamma (IFN-γ) is the cytokine most studied, and IFN-γ priming has been shown to upregulate the MSC expression of several immunosuppressive molecules, including IDO, cyclooxygenase 2 (COX-2), TGF-β, and HGF [9]. IFN-γ is also effective on the surface molecules of MSCs. It has been reported that immune regulatory surface molecule expressions, such as PD-L1, HLA-G, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), were increased with IFN-γ stimulation [6, 10, 11]. In addition to IFN-γ, tumor necrosis factor alpha (TNF-α), IL-6, and IL-17, may be used. Although not as much as IFN-γ, these cytokines have also been shown to cause an increase in IDO, PGE2, IL-10, and TGF-β expression [12–14].

T lymphocytes are responsible for the formation of the adaptive immune response, and require 3 different signals for activation. Signal 1 is provided by the T-cell receptor upon recognition of an antigen on MHC molecules. Signal 2 is generated by co-stimulatory molecules, such as CD80/CD86 (B7.1/B7.2), on antigen-presenting cells. The cytokines in the environment enable the third signal. If Signal 1 is not supported by Signal 2, anergy or tolerance against the presented antigen occurs [15]. MSCs are known to not express costimulatory molecules, and therefore induce anergy in T cells [16]. It has been reported that MSCs do not express MHC-II, but stimuli such as IFN-γ can increase expression without affecting the expression of the costimulatory molecules CD80/CD86 [17]. CD80 and CD86 co-stimulation plays a critical role in shaping the activity of T cells. However, in addition to naive T cells, various costimulatory molecules shape the activation of effector and memory T cells. CD137L (4-1BBL), CD252 (OX40L), CD274 (PD-L1), CD275 (inducible costimulator ligand [ICOSL]) have been studied extensively in the literature [18]. It is well known that the cytokines IFN-γ, IL-4, and IL-10 have direct effects on antigen presentation and shaping the T cell phenotype, co-stimulation in dendritic cells (DCs), and macrophage activation [19]. However, the current literature does not reveal how MSCs express alternative costimulatory molecules or how these expressions might change with cytokine stimulation. The objective of this study was to investigate alterations in the costimulatory molecule expression of MSCs preconditioned with inflammatory cytokines. Human adipose tissue (AD) MSCs and THP-1 macrophages were stimulated with IFN-γ, IL-4, and IL-10 cytokines, and the effects of these stimuli on the expression of HLA, CD80, CD86, CD137L, CD252, CD274, and CD275 molecules was evaluated. The costimulatory molecule expression of MSCs were compared in different inflammatory environments to THP-1 macrophages, a professional antigen-presenting cell (APC) model.

Materials and Methods

Cell culture

AD-MSCs (PCS-500-011; American Type Culture Collection, Manassas, VA, USA) and THP-1 cells (TIB-202; American Type Culture Collection, Manassas, VA, USA) were procured. The AD-MSCs were cultured using Dulbecco's Modified Eagles Medium F12 (DMEM-F12; Biosera, Inc., Manila, Philippines), and the THP-1 cells were cultured with Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum, and 100 U/mL penicillin, 100 µg/mL streptomycin and 1% 2mM L-glutamate (all Biosera, Inc., Manila, Philippines) at 37˚C and 5% CO2 in a cell culture incubator. The cells were checked daily and subcultured when they reached 70% to 80% confluence. Third-passage cells were used to perform the experiments.

Preconditioning

To create fully differentiated THP-1 macrophage cells, 3x105 cells/mL THP-1 cells were seeded in each well of a 6-well culture plate and stimulated with 10 ng/mL phorbol 12-myristate-13-acetate (PMA) (MilliporeSigma, Burlington, MA, USA) for 24 hours. Next, 3x105 AD-MSCs were seeded in each well of another set of 6-well culture plates and cultured for 24 hours. The medium was replaced and nonadherent cells were removed. To precondition the cells, 50 ng/mL IFN-γ, 40 ng/mL IL-4, and 40 ng/mL IL-10 (all Reprokine Ltd., Congers, NY, USA) were added to the appropriate wells and left to culture for 24 hours. The same number of cells were cultured as unconditioned controls. After incubation, the cells to be used for flow cytometry analysis were detached using Accutase solution (Biosera, Inc., Manila, Philippines), and the cells to be used in the quantitative polymerase chain reaction (qPCR) analysis were collected using a cell scraper. All of the experiments were performed in triplicate.

Flow cytometry

Anti-human CD86/B7-2 fluorescein isothiocyanate (FITC) (clone: BU63) and CD274/PD-L1 phycoerythrin (PE) (clone: 29E.2A3) fluorescent-labeled antibodies were purchased from EXBIO Praha, a.s., Vestec, Czech Republic. Anti-human CD80/B7-1 PE.Cy5 (clone: 1D10), CD137L/4-1BBL PE (clone: 5F4), CD252/OX40L PE (clone: 11C3.1), CD275/ICOSL (clone: 2D3), HLA-A,B,C FITC (clone: W6/32), and HLA-DR,DP,DQ FITC (clone: Tü39) fluorescent labeled antibodies were purchased from Biolegend Inc., San Diego, CA, USA. The IFN-γ, IL-4, and IL-10 stimulated cells and the unstimulated cells were stained according to the manufacturer's protocol and analyzed using an Accuri C5 flow cytometer (BD Biosciences, San Jose, CA, USA). FlowJo v10 software (FlowJo LLC, Ashland, OR, USA) was used to perform all of the analyses.

Quantitative polymerase chain reaction

Total RNA isolation was performed in accordance with the protocol provided for the Purelink RNA MiniKit (Cat. no: 12183018A; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and once the

complementary DNA was synthesized using the high-capacity RNA-to-cDNA kit (Cat. no: 4368814; Invitrogen Corp., Carlsbad, CA, USA). Forward and reverse primers of CD80/B7-1, CD86/B7-2, CD137L/4-1BBL, CD252/OX40L, CD274/PD-L1, CD275/ICOSL, HLA-ABC, and HLA-DR genes were purchased from Sentegen Biotech, Ankara, Turkey (Table 1). SYBR Green PCR Master Mix (Cat. no: 4344463; ThermoFisher Scientific, Inc., Waltham, MA, USA) was used to determine change in gene expressions and the reactions were assessed using the StepOnePlus Real-Time PCR System (Cat. no: 4376600 Applied Biosystems, Foster City, CA, USA). GAPDH was used as a reference gene, and the relative gene expression differences were calculated using the delta-delta cycle threshold (CT) method. The primers of the genes used in the qPCR analysis are shown in Table 1.

Statistical analysis

Data obtained from flow cytometry and qPCR analysis were evaluated using Prism v. 7.0 software (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk test was used to analyze the distribution of data. Data with a normal distribution were compared using ordinary one-way analysis of variance, and those without normal distribution were evaluated using the Kruskal-Wallis method. Results of $p<0.05$ were considered statistically significant.

Results

Microscopic evaluation was performed to observe the effects of cytokines on cell morphology. IFN-γ and IL-10-stimulated THP-1 cells displayed spindle morphology, while the stimulated IL-4 cells and the cells that had not been stimulated and had a more round morphology. Cytokine stimuli did not lead to an observable difference in the morphology of MSCs. The alterations in the microscopic images of THP-1 and AD-MSCs following cytokine stimulation are shown in Figure 1.

Flow cytometry

Positive cell frequencies (PCFs) were evaluated using flow cytometry to observe molecule expression changes in un-

Table 1. Table of primers used in quantitative polymerase chain reaction analysis

Gene	Forward	Reverse
CD80 (B7-1)	AGGAACACCCTCCAATCTCTG	GGTCAAAAGTGAAAGCCAACA
CD86 (B7-2)	CTGCTCATCTATACACGGTTACC	GGAAACGTCGTACAGTTCTGTG
CD137L (4-1BB)	TCAGGCTCCGTTTCACTTG	CAGGTCCACGGTCAAAGC
CD252 (OX40L)	TGATGACTGAGTTGTTCTGCACC	CCTACATCTGCCTGCACTTCTC
CD274 (PD-L1)	TATGGTGGTGCCGACTACAA	TGCTTGTCCAGATGACTTCG
CD275 (ICOSL)	CCCAGGACGAGCAGAAGTTT	TGAAGTTTGCTGCCACATGC
HLA-ABC	TGGGAGCTGTCTTCCCAGCCC	CCACATCACGGCAGCGACCA
HLA-DR	AGACAAGTTCACCCCACCAG	AGCATCAAACTCCCAGTGCT
GAPDH	GCCGCATCTTCTTTTGCGTC	GACGAACATGGGGGCATCAG

CD: Cluster of differentiation; GAPDH: Gliseraldehid 3-Fosfat Dehidrogenaz; HLA: Human leukocyte antigen; ICOSL: Inducible T cell costimulatory ligand; PD: Programmed death ligand-1.

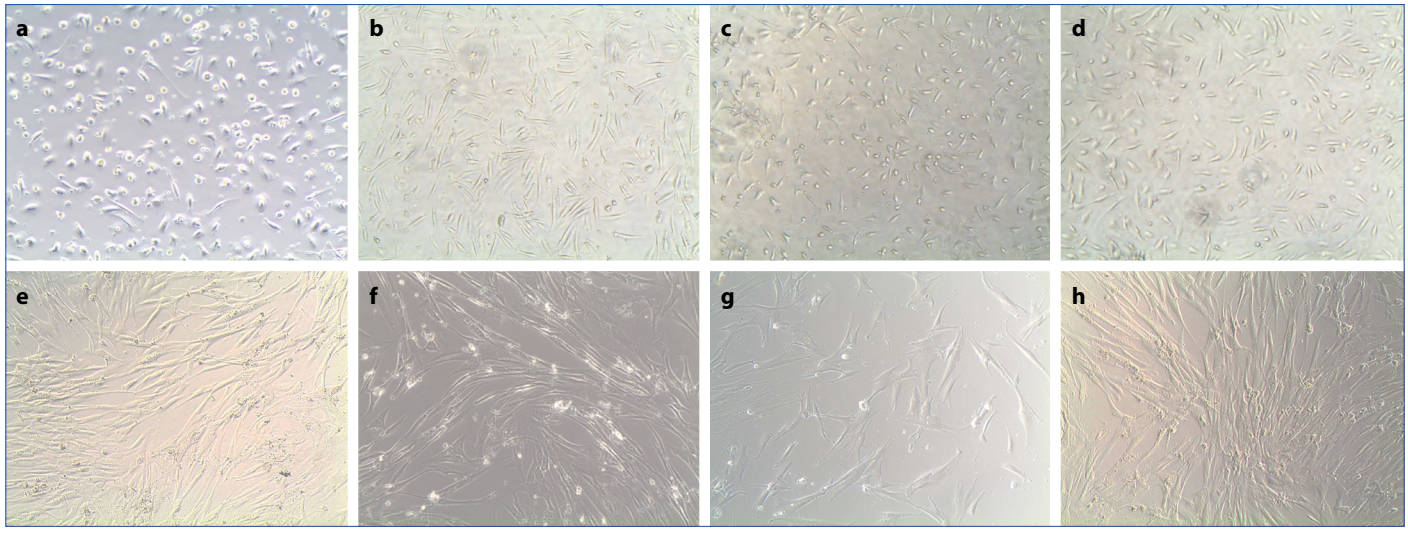

Figure 1. Microscopy images of (a) unconditioned, (b) preconditioned with interferon gamma (IFN-γ), (c), interleukin (IL) 4, (d) IL-10 human acute monocytic leukemia cell line (THP-1) macrophages, and (e) unconditioned, (f) preconditioned with IFN-γ, (g) IL-4, (h) and IL-10 adipose tissue mesenchymal stem cells.

conditioned and cytokine-preconditioned cells. Histogram graphs of the flow cytometry analysis are shown in Figure 2, and group comparison charts are provided in Figure 3. The PCFs obtained from flow cytometry analysis of all groups are summarized in Table 2. It was observed that the frequencies of CD80 (p=0.0003), CD86 (p<0.0001), CD137L (p<0.0001), CD252 (p=0.0003), CD274 (p=0.0077), CD275 (p<0.0001), and HLA-II (p<0.0001) PCFs of THP-1 macrophages were significantly higher than those of the AD-MSCs, while HLA-I demonstrated no significant difference (p=0.1506) (Fig. 3).

It was also noted that cytokine stimuli applied to THP-1 macrophages created significant changes in PCFs. The frequencies of CD80, CD86, CD252, CD274, and CD275 PCFs of THP-1 macrophages significantly increased with IFN-γ (p<0.0001, p=0.0181, p=0.0010, p<0.0001, and p<0.0001 respectively), but significantly decreased with IL-10 (p=0.0004, p<0.0001, p<0.0001, p=0.0011, and p<0.0001 respectively). IL-4 stimulation significantly increased the CD86 (p<0.0001) and CD274 (p<0.0001) PCFs, but significantly reduced the CD80 (p=0.0012) PCFs and had no significant effect on other costimulatory molecules.

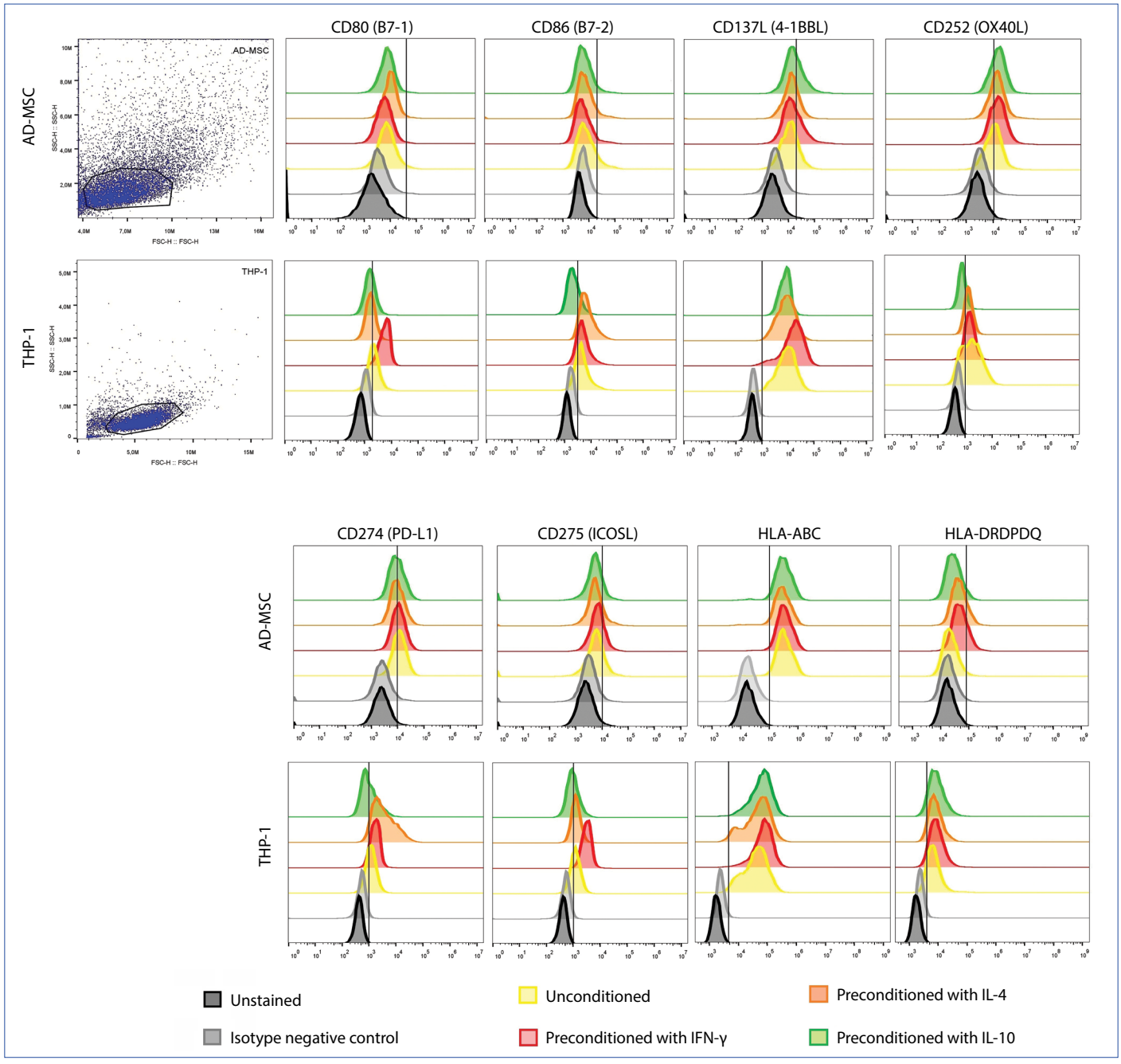

Figure 2. Histogram graphs obtained from flow-cytometry analysis of human acute monocytic leukemia cell line (THP-1) macrophages and adipose tissue mesenchymal stem cells (AD-MSCs).

HLA: Human leukocyte antigen; ICOSL: Inducible T cell costimulator ligand; IFN-γ: Interferon gamma; IL: Interleukin; PD-L1: Programmed death-ligand.

Table 2. Table of positive cell frequencies obtained from flow cytometry analysis of all groups

	THP-1 macrophages				AD-MSCs			
	US	IFN-γ	IL-4	IL-10	US	IFN-γ	IL-4	IL-10
CD80 (B7-1)	52.3±7.56	92.9±0.92	28.3±4.23	24.6±3.70	0.86±0.13	0.06±0.04	0.68±0.11	0.21±0.05
CD86 (B7-2)	69.1±2.28	79.0±4.17	89.0±2.57	20.5±2.96	12.0±1.67	5.84±0.8	8.40±1.16	6.1±0.84
CD137L (4-1BBL)	97.7±1.25	96.2±1.20	97.5±1.05	97.5±1.51	18.9±2.6	30.5±4.2	19.81±2.7	29.9±3.2
CD252 (OX40L)	66.0±2.51	76.9±1.76	72.3±2.77	17.8±0.53	45.4±1.9	67.6±1.7	63.2±2.6	68.9±2.6
CD274 (PD-L1)	61.8±3.50	87.0±3.17	91.9±2.92	44.2±4.05	51.6±0.66	54.9±1.27	42.35±1.63	42.9±1.29
CD275 (ICOSL)	65.6±3.01	95.4±0.96	70.3±2.88	41.8±1.35	10.8±0.35	21.2±0.83	10.9±0.80	8.47±0.64
HLA-I	95.5±1.49	96.7±1.43	95.1±1.67	96.6±1.35	93.6±1.12	93.4±1.02	90.9±1.18	93.1±0.89
HLA-II	81.3±1.40	88.0±1.48	87.2±1.48	86.1±1.49	0.94±0.27	18.2±2.27	13.6±1.71	2.92±0.43

The data in the table are listed as mean and SD and were obtained from 3 independent experiments. AD-MSCs: Adipose-derived mesenchymal stem cells; HLA: Human leukocyte antigen; ICOSL: Inducible T cell costimulator ligand; IFN-γ: Interferon gamma; IL: Interleukin; PD-L1: Programmed death-ligand 1; THP-1: Human acute monocytic leukemia cell line; US: Unstimulated.

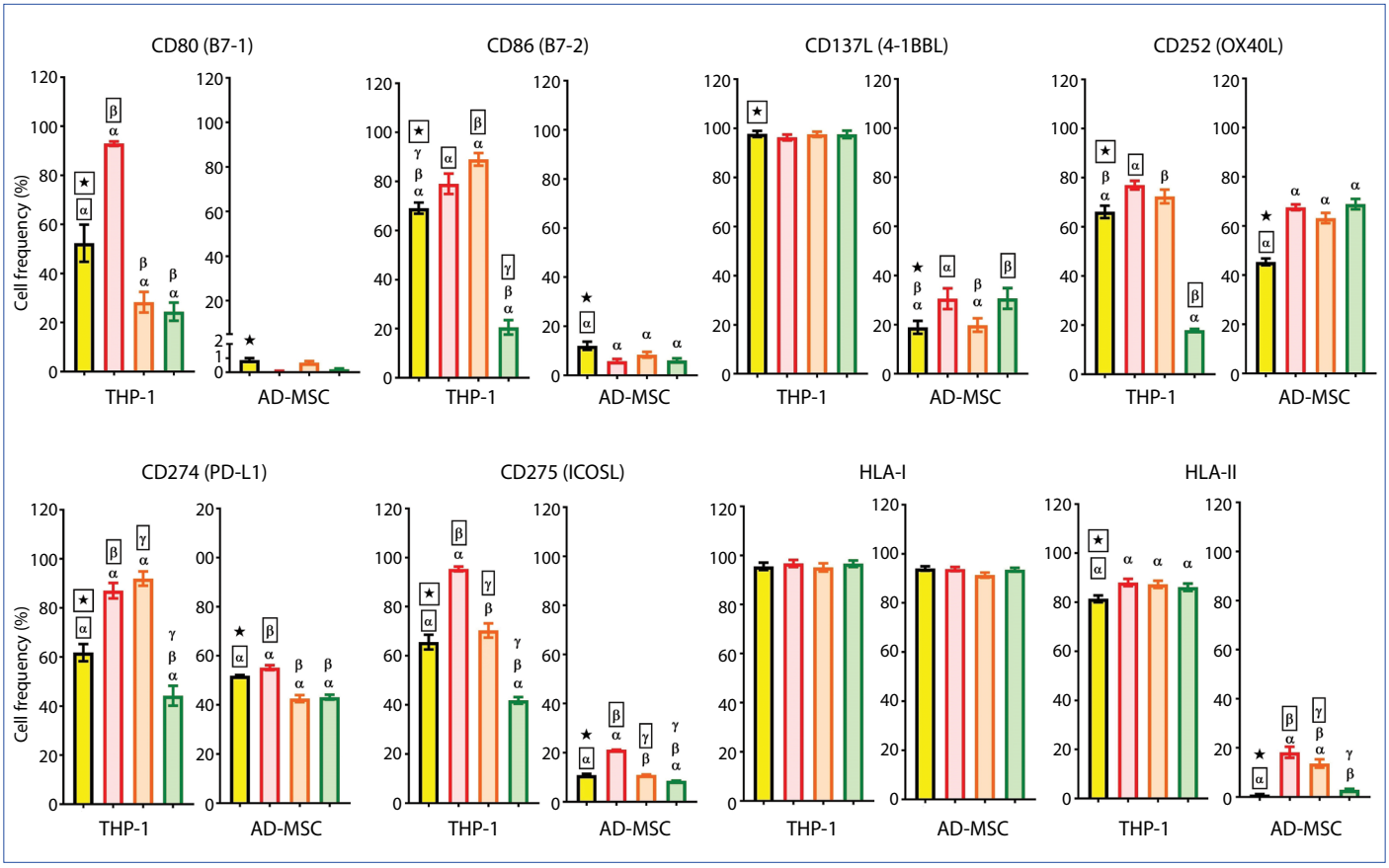

Figure 3. Comparison charts of unconditioned (yellow), preconditioned with interferon gamma (IFN-γ) (red), interleukin (IL) 4 (orange), and IL-10 (green)-positive cell frequencies obtained from flow cytometry analysis of all groups. The data are presented as mean and SD. There is a statistically significant difference ($p<0.05$) between the columns marked with the same symbol shown in the box.

Compared to THP-1 macrophages, a relatively very small population of AD-MSCs was positive for CD80, CD86, CD137L, CD275 and HLA-II (Fig. 3). However, it was observed that the cytokine stimuli caused statistically significant changes in these molecule expressions. CD86 PCFs were significantly decreased by all cytokines ($p=0.0009$, $p=0.0212$, and $p=0.0011$ respectively), and CD137L PCFs were significantly increased with IFN-γ ($p=0.0104$) and IL-10 ($p=0.0136$). CD252 PCFs were significantly increased by all cytokines ($p<0.0001$, $p<0.0001$, and $p<0.0001$ respectively). CD274 PCFs were significantly decreased by IL-4 ($p<0.0001$) and IL-10 ($p=0.0001$), but increased by IFN-γ ($p=0.0483$). CD275 PCFs were significantly decreased by IL-10 ($p=0.0114$), but in-

Table 3. Table of positive cell frequencies obtained from flow cytometry analysis of all groups

	THP-1 Macrophages				AD-MSCs			
	US	IFN-γ	IL-4	IL-10	US	IFN-γ	IL-4	IL-10
CD80 (B7-1)	52.3±7.56	92.9±0.92	28.3±4.23	24.6±3.70	0.86±0.13	0.06±0.04	0.68±0.11	0.21±0.05
CD86 (B7-2)	69.1±2.28	79.0±4.17	89.0±2.57	20.5±2.96	12.0±1.67	5.84±0.8	8.40±1.16	6.1±0.84
CD137L (4-1BBL)	97.7±1.25	96.2±1.20	97.5±1.05	97.5±1.51	18.9±2.6	30.5±4.2	19.81±2.7	29.9±3.2
CD252 (OX40L)	66.0±2.51	76.9±1.76	72.3±2.77	17.8±0.53	45.4±1.9	67.6±1.7	63.2±2.6	68.9±2.6
CD274 (PD-L1)	61.8±3.50	87.0±3.17	91.9±2.92	44.2±4.05	51.6±0.66	54.9±1.27	42.35±1.63	42.9±1.29
CD275 (ICOSL)	65.6±3.01	95.4±0.96	70.3±2.88	41.8±1.35	10.8±0.35	21.2±0.83	10.9±0.80	8.47±0.64
HLA-I	95.5±1.49	96.7±1.43	95.1±1.67	96.6±1.35	93.6±1.12	93.4±1.02	90.9±1.18	93.1±0.89
HLA-II	81.3±1.40	88.0±1.48	87.2±1.48	86.1±1.49	0.94±0.27	18.2±2.27	13.6±1.71	2.92±0.43

The data in the table are listed as mean and SD and were obtained from 3 independent experiments. AD-MSCs: Adipose-derived mesenchymal stem cells; HLA: Human leukocyte antigen; ICOSL: Inducible T cell costimulator ligand; IFN-γ: Interferon gamma; IL: Interleukin; PD-L1: Programmed death-ligand 1; THP-1: Human acute monocytic leukemia cell line; US: Unstimulated.

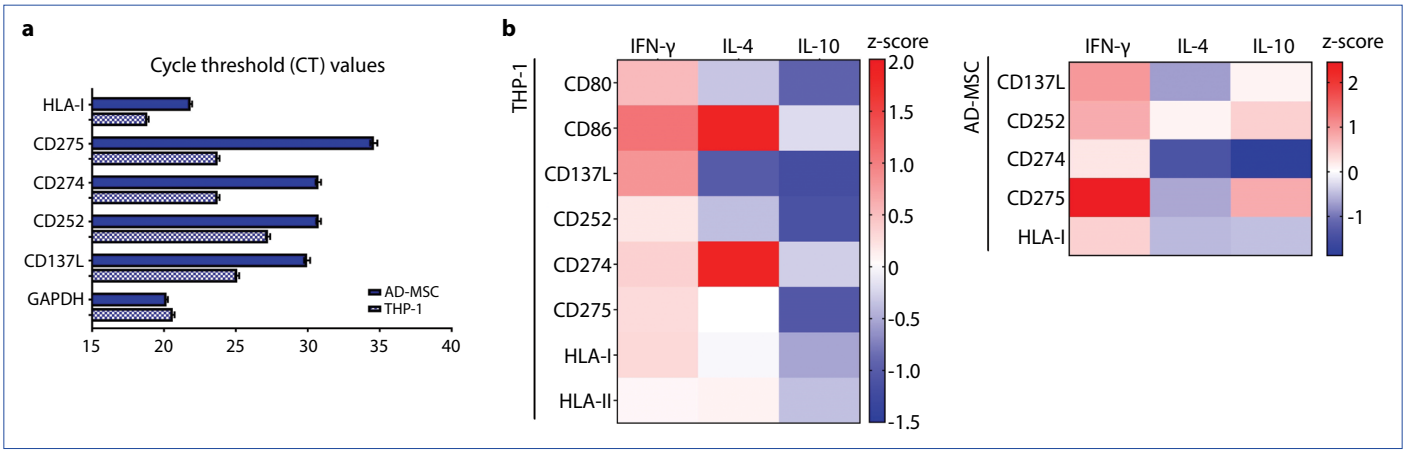

Figure 4. Comparison graphs of data from quantitative polymerase chain reaction analysis of all groups. (a) Comparison of cycle threshold data of gene expressions that can be measured in both human acute monocytic leukemia cell line (THP-1) macrophages and adipose tissue mesenchymal stem cells (AD-MSCs). (b) Heat-map graphs of the changes of the genes expressed in THP-1 macrophage and AD-MSCs with interferon gamma (IFN-γ), interleukin (IL) 4, and IL-10 stimuli. (delta-delta cycle threshold values were presented by converting to z-score.)
HLA: Human leukocyte antigen; IFN-γ: Interferon gamma; IL: Interleukin.

creased by IFN-γ ($p<0.0001$). HLA-II PCFs were significantly increased by only IFN-γ ($p<0.0001$) and IL-4 ($p<0.0001$).

Gene expressions

Since the CT values of CD80, CD86, and HLA-II genes of AD-MSCs are >35, these molecules were excluded from evaluation. The GAPDH CT values of unstimulated THP-1 and AD-MSCs were similar, but the CT values of MSCs were significantly higher for other molecules (Fig. 4a). This finding suggested that the gene expressions of THP-1 cells were significantly higher than those of MSCs for the molecules evaluated. When the changes caused by cytokines were evaluated, it was observed that IFN-γ significantly increased all gene expressions in the THP-1 cells, an in contrast, IL-10 significantly reduced all gene expressions. CD86 and CD274 expressions of THP-1 cells were significantly increased by IL-4, but expressions of other molecules were decreased significantly (Fig. 4b). Similarly, IFN-γ significantly increased overall gene expression of AD-MSCs. IL-4 significantly reduced the expression of genes other than CD252. IL-10 significantly reduced CD274 and HLA-I expressions, but increased CD137L, CD252, and CD275 (Fig. 4b). The delta-delta CT values of all of the groups are summarized in Table 3. The p values of the statistical comparisons are presented in the Table 4.

Discussion

This study was an investigation of the expression of costimulatory molecules, which have critical roles of the activation of T cells in AD-MSCs. We used macrophages differentiated from THP-1 cells as the reference APC model. It was found that the CD80, CD86, CD137L, CD252, CD274, CD275, and HLA-II PCFs of AD-MSCs were significantly lower than those of THP-1 macrophages; however, HLA-I revealed no significant difference. We found that preconditioning with IFN-γ led to a significant

Table 4. One-way analysis of variance with Tukey's multiple comparison test results of the delta-delta cell threshold values of all groups

THP-1 macrophages					
Compared groups	**Adjusted p value**	**Compared groups**	**Adjusted p value**	**Compared groups**	**Adjusted p value**
CD80 (B7-1)		**CD252 (OX40L)**		**HLA-ABC**	
US vs. IFN-γ	<0.0001	US vs. IFN-γ	<0.0001	US vs. IFN-γ	<0.0001
US vs. IL-4	<0.0001	US vs. IL-4	<0.0001	US vs. IL-4	>0.9999
US vs. IL-10	<0.0001	US vs. IL-10	<0.0001	US vs. IL-10	<0.0001
IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	<0.0001
IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001
IL-4 vs. IL-10	<0.0001	IL-4 vs. IL-10	<0.0001	IL-4 vs. IL-10	<0.0001
CD86 (B7-2)		**CD274 (PD-L1)**		**HLA-DRDPDQ**	
US vs. IFN-γ	<0.0001	US vs. IFN-γ	<0.0001	US vs. IFN-γ	0.5511
US vs. IL-4	<0.0001	US vs. IL-4	<0.0001	US vs. IL-4	0.0664
US vs. IL-10	0.0466	US vs. IL-10	<0.0001	US vs. IL-10	<0.0001
IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	>0.9999
IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001
IL-4 vs. IL-10	<0.0001	IL-4 vs. IL-10	<0.0001	IL-4 vs. IL-10	<0.0001
CD137L (4-1BBL)		**CD275 (ICOSL)**			
US vs. IFN-γ	<0.0001	US vs. IFN-γ	<0.0001		
US vs. IL-4	<0.0001	US vs. IL-4	>0.9999		
US vs. IL-10	<0.0001	US vs. IL-10	<0.0001		
IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	<0.0001		
IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001		
IL-4 vs. IL-10	0.0076	IL-4 vs. IL-10	<0.0001		
Adipose mesenchymal stem cells					
CD137L (4-1BBL)		**CD252 (OX40L)**		**CD274 (PD-L1)**	
US vs. IFN-γ	<0.0001	US vs. IFN-γ	<0.0001	US vs. IFN-γ	<0.0001
US vs. IL-4	<0.0001	US vs. IL-4	<0.0001	US vs. IL-4	<0.0001
US vs. IL-10	<0.0001	US vs. IL-10	<0.0001	US vs. IL-10	<0.0001
IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	<0.0001
IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001
IL-4 vs. IL-10	<0.0001	IL-4 vs. IL-10	<0.0001	IL-4 vs. IL-10	<0.0001
CD275 (ICOSL)		**HLA-ABC**			
US vs. IFN-γ	<0.0001	US vs. IFN-γ	<0.0001		
US vs. IL-4	0.0107	US vs. IL-4	0.9996		
US vs. IL-10	<0.0001	US vs. IL-10	>0.9999		
IFN-γ vs. IL-4	<0.0001	IFN-γ vs. IL-4	<0.0001		
IFN-γ vs. IL-10	<0.0001	IFN-γ vs. IL-10	<0.0001		
IL-4 vs. IL-10	<0.0001	IL-4 vs. IL-10	0.9908		

HLA: Human leukocyte antigen; ICOSL: Inducible T cell costimulator ligand; IFN-γ: Interferon gamma; IL: Interleukin; PD-L1: Programmed death-ligand 1THP-1: Human acute monocytic leukemia cell line; US: Unstimulated.

increase in all of the molecule expressions evaluated in THP-1 macrophages, but IL-10 led to a significant decrease in the opposite direction. IL-4 caused a significant decrease in the CD80 PCFs of THP-1 cells, and a significant increase in the CD86 and CD274 PCFs. In addition, we observed that the CD80, CD86, and HLA-II PCFs of AD-MSCs were extremely when low compared with THP-1 cells. We found that IFN-γ led to a significant increase in CD137L, CD252, CD274, and CD275 mRNA and PCFs in AD-MSCs, similar to THP-1 cells.

Three basic signals have been identified in the activation of T cells by antigen presentation. The first signal (Signal 1) for antigen-specific activation of T cells is provided by the interaction of the T cell receptor and peptide-HLA complexes. Antigen presentation via HLA-I is restricted to CD8 T cells, and HLA-II is restricted to CD4 T cells. Therefore, while HLA-I is expressed in all cells, HLA-II is expressed by professional antigen presenting cells (APCs) [20]. We observed that almost all of the THP-1 macrophages and AD-MSCs were positive for HLA-I.

Unlike THP-1 macrophages, we found that AD-MSCs did not express HLA-II; however, we detected a small but significant increase with IFN-γ and IL-4 stimulation. This finding indicated that AD-MSCs may not present antigens to CD4 T cells.

Signal 1 alone is not sufficient for T cell activation and lineage commitment. A second signal (Signal 2) provided by costimulatory molecules is necessary for complete activation [20]. CD80/B7-1 and CD86/B7-2 are costimulatory molecules that are highly expressed on macrophages and mature monocyte-derived DCs (mo-DCs) and interact with the CD28 molecule on the T cell surface, enabling T cell activation [21]. It has been reported that IFN-γ significantly increases both CD80 and CD86 expressions, IL-4 leads to a more pronounced increase for CD86, but IL-10 significantly reduces expression of both molecules [22]. Our results were consistent with the literature. CD80 and CD86 expressions of THP-1 macrophages were increased significantly by IFN-γ but were significantly decreased by IL-10. However, IL-4 significantly suppressed CD80 expression while significantly increasing CD86 expression. A limited number of studies of MSCs have shown that these cells do not express CD80 and CD86, and IFN-γ stimulation does not affect this condition [23]. Flow cytometry analysis indicated that AD-MSCs did not express CD80, and CD86 expressions were extremely low. QPCR analysis did not reveal expression of either molecule (Fig. 3 and 4).

CD137L/4-1BBL is a costimulatory molecule commonly expressed by APCs and interacts with CD137/4-1BB found in T and natural killer (NK) cells. The interaction of CD137/CD137L not only activates T and NK cells, but APCs are differentiated in the pro-inflammatory direction [24]. A review of the current literature did not disclose another study investigating the effects of different cytokine stimuli on CD137L expression in macrophages or mo-DCs. We found that more than 90% of THP-1 macrophages were positive for CD137L, and that CD137L mean fluorescent intensity (MFI) values and mRNA expressions only increased significantly with IFN-γ (Fig. 3 and 4). Only 1 study has demonstrated that MSCs can express CD137L and suppress T cell proliferation [25]. We found that AD-MSCs had a significantly lower but basal CD137L expression compared with THP-1 macrophages, and this expression was increased significantly by IFN-γ and IL-10 stimuli (Fig. 3 and 4).

CD252/OX40L interacts with the CD134/OX40 located on the T cell surface and produces a signal that increases the proliferation and survival of effector T cells. OX40 / OX40L interaction creates a bidirectional signal that activates both T cells and APCs, and activation of APCs allows them to express more OX40L [26]. There are only a few studies that have examined the OX40L expression of both APCs and MSCs and how they change with cytokine stimulation. It has been reported that OX40L expression of microglia cells increases with IFN-γ stimulation [27]. It has also been observed that MSCs increase regulatory T cell (Treg) ratios more effectively by overexpressing OX40L [28]. We found that the OX40L expression of THP-1 macrophages increased significantly with IFN-γ and IL-4 stimuli, but decreased significantly with IL-10. We identified OX40L expression in more than half of the AD-MSCs, and observed that expression was significantly increased by all three cytokines (Fig. 3 and 4).

CD274/PD-L1 interacts with CD279/PD-1 on the T cell surface, causing an increase in apoptosis and a decrease in activation and proliferation. This provides for the development of central and peripheral tolerance. Therefore, the PD-1/PD-L1 signal plays an important role in the pathogenesis of a wide range of diseases, such as chronic infection, autoimmune diseases, and cancer [29]. We found that the PD-L1 expression of THP-1 macrophages was increased significantly by IFN-γ and IL-4, but decreased significantly by IL-10. We observed that almost half of the AD-MSCs expressed PD-L1, and that this expression increased significantly with IFN-γ, but decreased significantly with IL-4 and IL-10.

CD275/ICOSL is the ligand of the CD278/ICOS molecule found in T cells and provides the effector and memory cells to proliferate and survive. It plays a critical role in antibody production, particularly by regulating follicular T cells [30]. ICOSL expression of DCs has been shown to increase significantly with IFN-γ and IL-4 [31]. It has been demonstrated that MSCs are able to express ICOSL and that the Treg induction capacity is proportional to the expressed ICOSL ratio [32]. We found that the ICOSL expression of THP-1 macrophages increased significantly with IFN-γ but decreased with IL-10. However, we also observed that in MSCs, ICOSL expression was limited to a small population, and this expression was significantly increased by IFN-γ, but decreased by IL-10.

The third and final basic signal in T cell activation is created by cytokines found in the environment, such as IFN-γ, IL-4, IL-10, or TGF-β [33]. MSCs could provide effective immune suppression through the powerful molecules they secrete; that is, MSCs are strong Signal 3- producer cells [2,3]. However, this effect is unfortunately limited by the presence of MSCs. Tolerogenic DCs produce suppressive molecules like MSCs, but unlike the MSCs, they play a critical role in the formation of peripheral tolerance because they express HLA-II [19]. MSCs have provided promising improvements in clinical trials examining immune pathologies such as graft-versus-host disease, systemic lupus erythematosus, and multiple sclerosis, which can be fatal and for which current treatment approaches are inadequate [34]. However, the results of these trials have shown that the effects of MSCs were temporary [35]. Therefore, it may be that the immunomodulation effects of MSCs are insufficient to establish an antigen-specific tolerance. Although the preconditioning approach improves the immunomodulation properties of MSCs, our findings indicate that this approach did not significantly change the costimulatory molecule expressions. This suggests that, since Signal 1 and Signal 2 were not properly generated by MSCs, they may be insufficient to formulate tolerance. HLA-I expression of MSCs could enable them to interact with CD8 T cells, but the absence of

costimulatory molecules will cause CD8 T cells to be anergic. MSCs need expression of HLA-II for anergy-inducing potential to occur on CD4 T cells. The transfer of HLA-II molecules to MSCs through gene engineering could enable these cells to form a broader and stronger tolerance, including CD4 T cells.

Conclusion

In conclusion, our data indicated that although the MSCs are potent immunomodulatory cells, the antigen presentation capabilities were not comparable to those of professional antigen presenting cells. In addition, the costimulatory molecule expressions of MSCs may not be significantly altered with a preconditioning approach. However, the transfer of costimulatory molecules through gene engineering could enable MSCs to develop a more effective and lasting tolerance potential.

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Technical Report

Data to assist in the determination of biochemistry test ranges to assess hemodialysis efficacy in patients with chronic renal failure

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Abstract

Objectives: Reference intervals are usually defined based on blood samples from healthy subjects and specific reference ranges for patients on hemodialysis (HD) are not currently available. The aim of the study was to establish expected ranges of biochemical analytes before and after HD for patients with chronic renal failure (CRF).

Methods: The findings of the 4 most recent quarterly check-ups of 684 patients (233 women and 451 men; age 18-95 years) treated with HD in several dialysis units attached to a single laboratory were studied. Biochemical analytes were measured using fully automated Roche Cobas C 501 or C 701 analyzers (Roche Diagnostics, Basel, Switzerland). Expected ranges were set according to International Federation of Clinical Chemistry and Clinical and Laboratory Standards Institute guidelines using the nonparametric method.

Results: Compared with pre-HD values, beta-2 microglobulin (β2m), chloride, creatinine, phosphate, potassium, and urea concentrations were lower post-HD ($p<0.001$), while bicarbonate, calcium, protein, and sodium concentrations were higher ($p<0.001$). Comparison with healthy subjects revealed that the levels of β2m, creatinine, and urea were higher before and after HD. Other analyte ranges were either lower, higher, or equivalent to healthy subjects in pre- and post-dialysis measurements. Differences between sexes were not significant, with the exception of creatinine, as well as a significant difference (>10%) in the creatinine level between individuals under and over 60 years of age ($p<0.0001$).

Conclusion: The establishment of specific ranges for dialysis patients could contribute to finding specific thresholds to monitor the effectiveness of HD.

Keywords: Biochemical, blood serum, hemodialysis, reference intervals, renal failure

The concept of a reference interval (RI) in human medicine was developed in the late 1960s by Grasbeck and Saris [1]. An RI is usually defined based on blood samples from healthy men and women, but similar studies of patients could contribute to finding specific values that provide a better ability to discriminate between states of health and disease.

Chronic renal failure (CRF) is characterized by a progressive loss of renal function [2]. CRF is associated with increased and decreased levels of some biochemical measurands. RIs based on healthy subjects are not optimal in this clinical context.

The objective of this study was to establish expected ranges for biochemical analytes commonly used in the monitoring before and after hemodialysis (HD) in a single laboratory.

Materials and Methods

Patients and sample collection

The subjects included in the study were male or female patients of a minimum 18 years of age with diagnosed CRF. A total of 684 patients (aged 18-95 years; 63% male, 37% female)

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with signs and symptoms of renal failure identified by expert nephrologists and confirmed by an estimated glomerular filtration rate (GFR) of <15 mL/min/1.73 sqm. The study period was January 2016 to December 2019. The samples analyzed were from the 4 most recent check-ups (2736 samples) and the separated serum was analyzed using routine methods.

Estimation of biochemical analytes

The biochemical analytes were measured using a fully automated Roche Cobas C 501 or C 701 analyzer (Roche Diagnostics, Basel, Switzerland). The methods and results are described in Table 1.

Statistical and data analysis

Data collection

Results were retrieved from the laboratory information system and statistical analysis was performed using XLSTAT software version 2018.1.1 (Addinsoft SARL, Paris, France). Retrospective data were used in accordance with the ethical standards of EU regulation 2016/676 on the protection of natural persons and the processing of personal data and the free movement of such data. The procedures used by our institution have been approved by French National Commission on Informatics and Liberty. All of the biochemical investigations were ordered by the treating physicians. The Labosud database is registered with the French National Commission on Informat-ics and Liberty, record no. 2073511v0.

Detecting and eliminating outliers

Dixon's range test [3], recommended by the CLSI [4] for statistical analysis in reference interval studies, was used to detect and eliminate extreme values as outliers. This test identifies the single most extreme value at the upper or lower limit as an outlier: after sorting the data into ascending order (smallest to largest), the ratio (Q_{exp}) was defined as the difference of the suspect value from its nearest one (D) divided by the range of the values (R). The obtained Q_{exp} value was compared to a critical Q-value (Q_{crit}) found in tables. This critical value should correspond to the confidence level (CL) we have decided to run the test (usually: CL=95%). The extreme value must be eliminated if Q_{exp} value was greater than Q_{crit}. XLSTAT software repeat application of criteria until no further observations are rejected.

Establishment of expected values

The expected values corresponded to the central 95th percentile based on nonparametric estimates defined by the 2.5th and 97.5th percentiles as the lower and upper reference limits, respectively. Reference limits with a confidence interval of 95% (95% CI) were estimated according to the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) recommendation [5]. The results were partitioned by age group and gender when the range exceeded the 95% CI.

Statistical analysis

The Mann-Whitney test was used to establish differences and the results were considered significant at $p<0.05$.

Results

The demographic profiles of the study patients are presented in Table 2. Expected values before and after hemodialysis were examined in all patients, by gender, and in 2 age groups (18-60 years, 61-93 years), and are summarized in Table 3. There was a significant difference in the serum creatinine and urea values between men and women ($p<0.0001$) and a significant difference (>10%) between patients who were older or younger than 60 years of age ($p<0.0001$). The pre-dialysis values of beta-2 microglobulin (β2m), creatinine, phosphate, potassium, and urea were significantly higher than the post-dialysis values and significantly lower for bicarbonate, calcium, chloride, total protein, and sodium ($p<0.0001$).

Table 1. Analytical and methodological characteristics of analytes with CVa data

Parameters measured in serum	Methods used	Reference standard	Imprecision (CVa %)
Bicarbonate	Enzymatic method using phosphoenolpyruvate carboxylase	Primary reference material	4.0
Beta-2 microglobulin	Immunoturbidimetric method	WHO reference	4.5
Calcium	Colorimetric method using 5 nitro 5' méthyl BAPTA	SRM 956 c level 2	1.6
Chloride	Indirect ion-selective electrode method	PC gravimetrically prepared	2.1
Creatinine	Enzymatic method	ID-MS	3.5
Phosphate	Ultraviolet phosphomolybdate method	Primary reference material	2.5
Potassium	Indirect ion-selective electrode method	PC gravimetrically prepared	1.2
Sodium	Indirect ion-selective electrode method	PC gravimetrically prepared	1.4
Total protein	Colorimetric biuret method	SRM 927	2.0
Urea	Kinetic test using urease and glutamate dehydrogenase	ID-MS	3.7

CVa: Analytical variation; ID-MS: Isotope dilution-mass spectrometry; PC: Primary calibrator; SRM: Standard reference material; WHO: World Health Organization.

Table 2. The age and gender distribution of the chronic renal failure patients

Age (years)	All patients		Female		Male	
	Number of patients	Number of results/analytes	Number of patients	Number of results/analytes	Number of patients	Number of results/analytes
18 - 30	15	60	5	20	10	40
31 - 40	25	100	7	28	18	72
41 - 50	29	116	11	44	18	72
51 - 60	70	280	23	92	47	188
61 - 70	189	756	62	248	127	508
71 - 80	188	752	63	252	125	500
81 - 90	156	624	56	224	100	400
>90	12	48	6	24	6	24
Total	684	2736	233	932	451	1804
Total %	100%		37%		63%	

Table 3. The upper (97.5th percentile) and lower (2.5th percentile) expected values before and after hemodialysis

Parameters	Age and gender	Before hemodialysis		After hemodialysis		Healthy subjects [14]
		Lower limit - 2.5th (95% CI)	Upper limit - 97.5th (95% CI)	Lower limit - 2.5th (95% CI)	Upper limit - 97.5th (95% CI)	
Beta-2 microglobulin (mmol/L)	Total patients	1234 (1195-1255)	3375 (3348-3414)	270 (249-288)	1528 (1492-1544)	68-186
	Female (F)	1302 (1248-1354)	3410 (3355-3461)	250 (220-274)	1405 (1373-1430)	
	Male (M)	1179 (1133-1212)	3552 (3314-3387)	286 (249-308)	1606 (1574-1633)	
	F :18-60 years	1099 (846-1087)	3614 (3501-3741)	250 (228-287)	1616 (1512-1746)	
	F: >60 years	1401 (1282-500)	3667 (3569-3835)	267 (222-323)	1519 (1445-1572)	
	M: 18-60 years	1084 (993-1137)	3741 (3643-3896)	280 (223-320)	1546 (1514-1643)	
	M: >60 years	1370 (1288-1459)	3512 (3429-3643)	268 (231-335)	1670 (1580-1723)	
Bicarbonate (mmol/L)	Total patients	17 (17-18)	28 (27-28)	22 (22-23)	31 (30-31)	22-29
Calcium (mmol/L)	Total patients	1.91 (1.90-1.92)	2.52 (2.51-2.53)	2.26 (2.26-2.27)	2.79 (2.78-2.80)	2.15-2.55
	Female (F)	1.93 (1.89-1.95)	2.53 (2.50-2.54)	2.26 (2.25-2.27)	2.78 (2.77-2.79)	
	Male (M)	1.89 (1.87-1.90)	2.52(2.51-2.54)	2.26 (2.26-2.27)	2.80 (2.79-2.81)	
	F: 18-60 years	2.00 (1.99-2.03)	2.52 (2.51-2.54)	2.25(2.24-2.26)	2.74 (2.73-2.75)	
	F: >60 years	1.78 (1.73-1.81)	2.55 (2.52-2.59)	2.31 (2.30-2.34)	2.83 (2.80-2.85)	
	M: 18-60 years	1.94 (1.92-1.96)	2.52 (2.51-2.54)	2.25 (2.24-2.26)	2.79 (2.78-2.80)	
	M: >60 years	1.81 (1.79-1.84)	2.49 (2.45-2.52)	2.26 (2.25-2.29)	2.88 (2 .86-2.90)	
Chloride (mmol/L)	Total patients	90 (90-91)	105 (104-105)	94 (94-95)	103 (102-103)	98-107
Creatinine (µmol/L)	Total patients	298 (291-302)	1071 (1063-1080)	71 (69-73)	368 (364-373)	<84
	Female (F)	251 (243-261)	970 (957-985)	60 (58-62)	297 (291-304)	
	Male (M)	325 (319-334)	1115 (1104-1125)	90 (88-92)	394 (389-400)	
	F: 18-60 years	238 (230-248)	885 (863-897)	58 (56-60)	275 (264-285)	
	F: >60 years	376 (355-400)	1144 (1111-1164)	82 (76-91)	376 (362-390)	
	M: 18-60 years	323 (316-332)	1024 (1011-1035)	88 (86-90)	392 (367-389)	
	M: >60 years	368 (355-390)	1316 (1289-1336)	101 (95-111)	509 (495-524)	
Phosphate (mmol/L)	Total patients	0.57 (0.56-0.58)	2.53 (2.51-2.56)	0.23 (0.22-0.24)	0.93 (0.92-0.95)	0.87-1.45
	Female (F)	0.57 (0.55-0.59)	2.49 (2.46-2.53)	0.22 (0.21-0.24)	0.83 (0.82-0.85)	
	Male (M)	0.56 (0.55-0.58)	2.54 (2.51-2.57)	0.25 (0.24-0.27)	0.99 (0.97-1.01)	
	F: 18-60 years	0.59 (0.57-0.63)	2.42 (2.36-2.48)	0.21 (0.20-0.23)	0.84 (0.81-0.86)	
	F: >60 years	0.50 (0.56-0.58)	2.93 (2.85-3.01)	0.28 (0.25-0.32)	0.88 (0.84-0.93)	
	M: 18-60 years	0.56 (0.55-0.58)	2.47 (2.42-2.52)	0.25 (0.24-0.27)	0.94 (0.91-0.97)	
	M: >60 years	0.61 (0.56-0.66)	3.09 (3.01-3.16)	0.27 (0.23-0.30)	1.25 (1.20-1.30)	
Potassium (mmol/L)	Total patients	3.5 (3.5-3.6)	6.5 (6.5-6.6)	2.8 (2.7-2.8)	4.5 (4.4-4.5)	3.5-5.1
	F: 18-60 years	3.5 (3.6-3.7)	6.5 (6.4-6.6)	2.8 (2.7-2.8)	4.4 (4.3-4.4)	

Table 3. Cont.

Parameters	Age and gender	Before hemodialysis		After hemodialysis		Healthy subjects [14]
		Lower limit - 2.5th (95% CI)	Upper limit - 97.5th (95% CI)	Lower limit - 2.5th (95% CI)	Upper limit - 97.5th (95% CI)	
	F: >60 years	3.8 (3.7-3.9)	6.7 (6.5-6.8)	2.7 (2.7-2.8)	4.5 (4.3-4.7)	
	M: 18-60 years	3.5 (3.4-3.5)	6.4 (6.4-6.5)	2.8 (2.8-2.9)	4.5 (4.5-4.6)	
	M: >60 years	3.6 (3.6-3.8)	6.7 (6.6-6.8)	2.8 (2.8-3.0)	4.6 (4.3-4.7)	
Protein total (g/L)	Total patients	56 (56-57)	79 (79-80)	59 (59-60)	89 (89-90)	64-83
	F: 18-60 years	56 (55-56)	77 (77-78)	58 (58-59)	86 (85-87)	
	F: >60 years	56 (56-57)	81 (79-82)	62 (61-63)	93 (92-94)	
	M: 18-60 years	55 (55-56)	79 (79-80)	59(59-60)	89 (89-90)	
	M: >60 years	57 (56-58)	80 (79-80)	62 (62-64)	92 (90-94)	
Sodium (mmol/L)	Total patients	131 (130-131)	144 (143-144)	135 (135-135)	144 (144-144)	132-146
	F: 18-60 years	131 (131-132)	144 (143-144)	135 (135-135)	144 (144-144)	
	F: >60 years	131 (131-132)	144 (144-145)	136 (134-136)	145 (145-146)	
	M: 18-60 years	132 (132-133)	144 (144-145)	135 (134-135)	144 (144-145)	
	M: >60 years	129 (128-130)	144 (144-145)	135 (134-135)	144 (144-145)	
Urea (mmol/L)	Total patients	8.3 (8.2-8.5)	32.8 (32.4-33.2)	1.3 (1.3-1.4)	10.2 (9.9-10.4)	3.2-8.1
	Female (F)	7.8 (7.6-8.1)	31.8 (31.6-32.4)	1.1 (1.0-1.1)	8.3 (7.7-8.7)	
	Male (M)	8.6 (8.5-8.8)	33.5 (32.9-33.9)	1.5 (1.4-1.6)	10.9 (10.5-11.2)	
	F: 18-60 years	7.7 (7.5-7.9)	30.5 (29.8-34.2)	1.1 (1.0-1.1)	7.1 (6.7-7.4)	
	F: >60 years	9.2 (8.3-10.7)	34.8 (32.9-35.9)	1.3 (1.2-1.5)	10.2 (9.7-12.8)	
	M: 18-60 years	8.5 (8.4-8.7)	32.8(32.0-33.3)	1.5 (1.4-1.5)	10.3 (9.9-10.7)	
	M: >60 years	8.9 (8.5-9.5)	35.3 (34.1-35.9)	1.5 (1.4-1.7)	12.6 (11.4-13.5)	

Parameters that differ by gender or age before or after HD are shown in bold and underlined. CI: Confidence interval; HD: Hemodialysis.

Discussion

Our results were consistent with those of numerous studies demonstrating biological disorders in the blood induced by continuous decrease in renal clearance or GFR [6-12]. Despite the effectiveness of dialysis in filtering and purifying the blood, we found that some analytes remained at very high levels and an interpretation of the result using the existing RIs was meaningless. In cases of renal failure, our results showed that the β2m level was about 18 times higher than the RI before dialysis and 8 times higher after dialysis. The serum level of β2m remained significantly elevated despite dialysis treatment. It would seem to be important for a laboratory to define expected values according to the practices of dialysis departments. The multiplicity of dialysis protocols could lead to greater variability of post-dialysis reference values. For example, the characteristics of the dialysis membrane or the duration of hemodialysis may be important factors influencing the level of β2m after dialysis [13]. The decrease in creatinine during dialysis is used to measure the effectiveness of the treatment. Although dialysis significantly reduces the serum creatinine level, it remains higher overall than that of healthy subjects (about 4 times higher). Defining post-dialysis reference values would be valuable as a means to alert the healthcare team to any changes, particularly if an increase is observed. Normal blood contains 3.2–8.1 mmol/L of urea [14]. Our results indicated that the minimum urea limit was lower after dialysis (~1.3 mmol/L). Excess urea was likely eliminated to prevent accumulation between dialysis sessions. When compared with healthy subjects, the post-dialysis results for calcium were higher, while phosphate was lower. According to some authors, hypocalcemia could precipitate adverse cardiac outcomes, such as cardiomyopathy, congestive cardiac failure, ventricular tachycardia, and other arrhythmias [15]. Furthermore, a high serum phosphate level could increase risk of mortality [16]. In this context, RIs appropriate for dialysis patients would be very useful to avoid the potential side effects of biochemical disturbances. As expected, we found that dialysis sessions led to a significant decrease in the plasma potassium concentration. According to some authors, an increase in dialysate potassium was associated with a smaller decrease in plasma potassium concentration, and accordingly, with a much lower prevalence of post-dialysis hypokalemia [17]. Our clinical data support the need to establish alert thresholds to prevent severe post-dialysis hypokalemia.

Conclusion

Ranges created specifically for dialysis patients could contribute to more efficient monitoring of HD treatment. These results could be adopted by laboratories using the same equipment with similar analytical performance and a patient population like the group evaluated in this study. For laboratories with dif-

ferent equipment or different patient demographic characteristics, transference of results published in this study should be performed following the protocol established by the CLSI [4].

Conflict of Interest: The authors declare that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Committee Approval: The procedures used by our institution have been approved by French National Commission on Informatics and Liberty.

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Review

The significance of teaching human biochemistry to dental students

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Abstract

Objectives: Biochemistry instructions in dental studies should highlight the fact that the basic biochemical processes occurring in the oral cavity are the same as those that take place in other tissues and organs of the body. Saliva is a complex fluid that plays an essential role in the maintenance of oral health and contains a wide range of proteins, enzymes (lysozyme, lactoferrin, peroxidases) and secretory immunoglobulins. Salivary cationic peptides and defense proteins, such as lysozyme, salivary amylase, cystatins, mucins, peroxidases, and statherin, are primarily responsible for innate immunity. The role of collagen, which forms a large part of the organic material of dentin, should be given prominence in the study of proteins. The study of carbohydrates and lipids is also an important topic, since the digestion of starch and lipids begins in the oral cavity due to the presence of various enzymes, e.g., amylase and lipase. The biochemistry syllabus for dental sciences should also include the role of weak acids and bases and buffers to help students understand the buffering capacity of saliva and its implications for oral health. Patients with poor glycemic control are more prone to oral manifestations of diabetes, including periodontal disease, salivary gland dysfunction, halitosis, burning mouth sensation, delayed wound healing, and increased susceptibility to infection. Diabetic patients are also at greater risk of an intraoperative diabetic emergency in the dental clinic. Therefore, dentists must appreciate and implement important dental management considerations when providing care to diabetic patients. Strategies to investigate bleeding disorders can help guide stepwise, rational testing for inherited and acquired causes of bleeding. It is clear that biochemistry is becoming increasingly important in the field of dentistry, and this should be reflected in both teaching and research.

Keywords: Biomarkers, biomolecules, clinical biochemistry, dental caries, medical biochemistry, oral cavity, saliva

Undergraduate students of medical sciences are required to apply professional and scientific knowledge in competence-based clinical skills acquired through laboratory training and patient interaction. Scientific and technological developments in medical sciences should be included in academic and clinical programs. Advancements in molecular biology, biochemistry, genomics, proteomics, and tissue engineering provide the basis for the use of new technologies in medicine. Laboratory sciences and research education is an important aspect of a health science curriculum and includes biomedical sciences and clinical biochemistry [1]. Knowledge of advanced medical biochemistry and laboratory clinical biochemistry is now an important aspect of medical and surgical disciplines (Fig. 1). The clinical biochemistry contribution of the analysis of glycated hemoglobin (HbA1c) to the diagnosis and monitoring of diabetes mellitus (DM) is particularly valuable. The importance of a serum lipid profile, including cholesterol and lipoproteins, for public health has increased enormously, as it identifies a risk factor for cardiovascular disease. Point-of-care testing, i.e., the development of a wide range of portable instruments, analyzers, and test kits, which allows for emergency testing of patients in hospitals or self-testing by individuals, was a substantial methodological development [2].

Biochemistry in dentistry

Medical sciences and therapeutics have progressed enormously over the last half a century, including the subject

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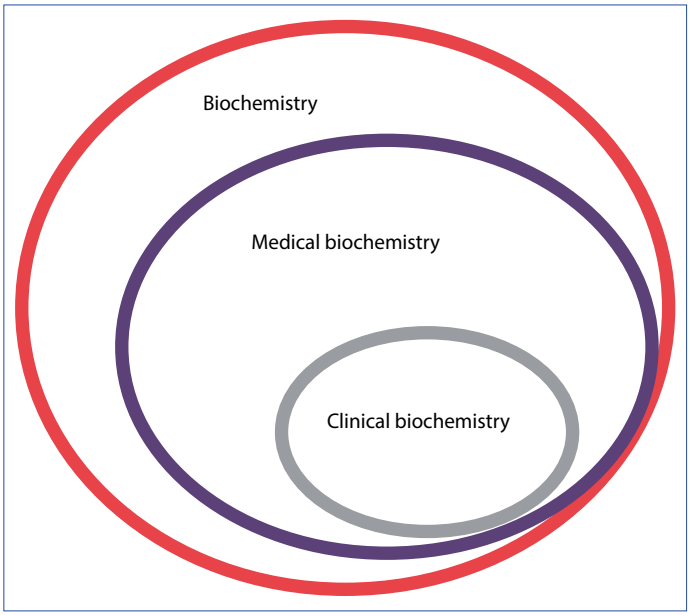

Figure 1. The relationship between general biochemistry, medical biochemistry, and clinical biochemistry (reproduced from Dominiczak, 2011).

area of oral health and dental diseases. The discipline of dentistry or dental hygiene was initially developed in the late 19th century to treat dental caries, but it eventually led to recognition and acceptance for the treatment of diseases of the oral cavity, also called the buccal cavity. It is now an advanced and well-established branch of human health sciences responsible for the treatment and management of diseases of the mouth, teeth, and related tissues. Better oral care and hygiene, advanced dental replacement materials, water fluoridation, and the adoption of drugs used for non-dental diseases have resulted in better dental treatments. The most common dental diseases, i.e., tooth cavities (dental caries) and bleeding gums (periodontal disease), are chronic conditions that are still not fully understood at the molecular and cellular level [3, 4]. Therefore, molecular biology and biochemistry have become increasing significant in dental sciences in order to understand the cause and pathogenicity of dental diseases [4].

The oral cavity is the uppermost part of the digestive system of human body and is the area of the mouth demarcated by the lips, cheeks, palate, and the floor of the cavity. The main structures of the mouth are the teeth, which tear and grind ingested food into small pieces suitable for digestion; the tongue, which mixes food and has sensory receptors for taste; and the palate, which separates the mouth from the nasal cavity, allowing separate passage of air and food. The favorable environment and the secretions present in the mouth help to soften food, facilitate swallowing, and start the process of digestion [5]. Moreover, oral fluids, such as saliva and oral tissue exudate, known as gingival crevicular fluid (GCF), are important diagnostic tools for the detection of diseases of the oral cavity. GCF is a useful body fluid for the diagnosis of DM and other inflammatory conditions [1]. Hence, it should be an important aspect of biochemistry to emphasize that the basic biochemical processes occurring in the oral cavity are the same as those that take place in other tissues and organs of the body [4].

Biochemistry of dental caries and plaque

The study of carbohydrates is an important topic in dental sciences since sugars have an significant role in the health of teeth. In addition, the digestion of carbohydrates (starches) starts in the mouth due to the presence the α-amylase enzyme, which is present in salivary secretions. Carbohydrates, such as polysaccharides, are classified according to their structure and function; storage polysaccharides are grouped as intracellular (e.g., starch and glycogen) or extracellular (e.g., dextran). Dextran is an intracellular polysaccharide found in cariogenic bacteria, while glycogen is present in the liver and skeletal muscles in humans. The study of complex polysaccharides constitutes an important area of interest to dental students, e.g., salivary mucins are glycoproteins, and the connective tissue and the organic matrix of dentine consists of mucopolysaccharides (glycosaminoglycans) [6]. The concentration of dietary sucrose has a significant contribution to dental caries and has the highest cariogenic potential. The Maillard reaction involves the biochemical interactions between sugars and proteins that are responsible for the lesion discoloration in the carious process, known as the browning reaction, categorized as a non-enzymatic glycation process. It involves an irreversible alteration in the amino acid structure leading to a change in protein function. The Maillard reaction leads to the formation of brown polymers called melanoidins, and the severity of disease can be evaluated based on the extent of brown discoloration [7]. Dental caries, or tooth decay, is a dynamic process in which the dental biofilm (microbial deposit) on the tooth surface undergoes metabolic reactions that result in chemical dissolution of the tooth. The frequency of carbohydrate consumption plays a vital role in the carious process. People who consume a large amount of carbohydrates have a lower oral pH since the buffering capacity of saliva is modified. A pH level of 5.5 is critical for the enamel in the carious process, as a lower pH indicates that there is net mineral loss of tissue from the tooth. Dentine is composed of more organic material and water, and also degrades in the carious process at a near neutral pH (pH 6.2) [7]. A carious tooth is the result of the dissolution of hydroxyapatite (HA) from calcified parts of the tooth accompanied by breakdown of the dentine collagen or enamel amelogenins, since the pH of the mouth is lowered due to anaerobic breakdown of carbohydrates by oral bacteria [8].

Matrix metalloproteinases (MMPs) are zinc-dependent, host-derived proteolytic enzymes, which are responsible for the degradation of the organic matrix of dentine in the carious process. The pH change in a carious lesion activates

MMPs, which function at a neutral pH, since saliva neutralizes an acidic pH, allowing MMPs to become activated and leading to the degradation of the organic matrix of the dentine. The MMPs require zinc, since it influences the signaling pathway of MMPs resulting in dentine remineralization. Therefore, zinc is an important element that can be used as a therapeutic agent for the remineralization of a tooth. Zinc-leaching dental materials, such as amalgams and cements (zinc phosphate, calcium hydroxide, zinc oxide), are thought to inhibit the demineralization of dentine and promote remineralization [7]. The production of organic acids (H^+) as a result of bacterial degradation of sugars causes the pH of plaque to fall, resulting in the dissolution of HA into calcium ions, hydrogen phosphate ions and water, thereby leading to demineralization within the enamel. It is well established that fluoride ions can inhibit bacterial growth. It adheres to the enamel, forming fluorohydroxyapatite, which is more resistant to acid attack. The antimicrobial property of fluoride inhibits enzyme enolase in the glycolytic pathway and bacteria are no longer able to thrive and promote tooth demineralization in the carious process [7].

Dental plaque biofilm is composed of diverse microflora found on the surface of teeth. The microorganisms within the biofilm metabolize fermentable carbohydrates in the diet, producing organic acids which dissolve the HA component of teeth. The bacteria are able to withstand and flourish in a low pH environment, and are subsequently responsible for the development of dental caries [7]. *Streptococcus mutans* is the principal bacterial species of dental caries and is able to thrive and multiply due to its ability to tolerate an acidic environment. The *S. mutans* glucosyltransferases (GTFs) are important enzymes in the formation and development of dental plaque biofilm. Specific GTFs are upregulated in response to a low pH, aiding in the colonization of other bacteria and the cohesion of plaque, contributing to a caries-favorable environment [7].

Biochemistry of saliva

Saliva is an important oral fluid that is secreted from the salivary glands and is involved in numerous functions in the mouth, including digestion, swallowing, lubrication, protection, and maintenance of tooth integrity [9-12]. The biochemistry of saliva is an important field of study and the presence of various organic and inorganic components have been identified. Saliva is an exocrine secretion consisting of 95% to 99% water and contains electrolytes, proteins, enzymes, immunoglobulins, antimicrobial factors, urea, mucosal glycoproteins, etc. that are important to oral health [13]. Saliva contains a variety of proteins with antibacterial properties and those necessary for inhibiting the spontaneous precipitation of calcium and phosphate ions in the salivary glands and their secretions. The balance of hydrogen-bicarbonate ions in saliva is responsible for the buffering capacity and pH that protect the enamel from dental caries. The action of buffers such as inorganic phosphate (Pi) and the carbonic acid-bicarbonate (H_2CO_3, HCO_3^-) system maintain a neutral pH in saliva. An increased salivary flow influences the biochemistry of the carious process, as it increases the concentration of bicarbonate, chloride, and sodium ions. The bicarbonate ion concentration neutralizes acids and promotes remineralization of teeth. Therefore, an adequate flow of saliva is necessary for the prevention of dental caries [7].

Sufficient salivary flow and composition are important for the lubrication and protection of oral tissue against desiccation, penetration, ulceration, and potential carcinogens by mucin and anti-proteases [14]. Saliva includes various components, including histatins, anti-bacterial polypeptides, acidic proline-rich proteins (PRPs), mucins, glycoproteins responsible for the viscosity of mucosal secretions, lysozyme, secretory immunoglobulin A (sIgA), cystatins, α-amylase, kallikreins, etc. [15-17]. The complex polysaccharides unique to the oral cavity include glycoproteins, such as salivary mucins, mucopolysaccharides (glycosaminoglycans) and extracellular polysaccharide (e.g., dextrans) occurring in cariogenic bacteria [8]. The oral cavity is responsible for the initial digestion of food or the formation of a bolus, which occurs largely due to the presence of saliva and digestive enzymes secreted by the salivary glands [4]. The digestion of polysaccharides (starches) starts in the oral cavity due to the presence of enzyme α-amylase in salivary secretions. This amylase has also been known as ptyalin, but the starch digestive enzyme present in the oral cavity is now commonly known as salivary amylase [4]. The main carbohydrate-digesting enzyme present in the saliva is α-amylase which digests or breakdowns starch into maltose, maltotriose, and dextrine, and is inactivated by the acidic environment of the gastrointestinal tract [4]. Its biological function is to digest or break down starch into maltose, maltotriose, and dextrin. The majority of this enzyme is synthesized in the parotid glands and the remainder in the submandibular glands, contributing about 50% of the total salivary proteins produced by the glands [13]. Saliva in the oral cavity is typically the first point of contact with any ingested substance, and its composition may be influenced by medication, oral lesions, intracellular diffusion, proteolytic enzymes derived from the host, oral micro-organisms, exercise, and circadian patterns.

Salivary diagnostics in clinical biochemistry

As a result of the rapid progress in salivary studies, the concept of salivaomics, the analysis of proteins, RNA, DNA, lipids, carbohydrates, metabolites and microorganisms in saliva, is growing. It is now recognized that salivary biomolecules can assist in the early diagnosis of several oral and systemic diseases [18]. Developments in salivaomics have also led to recognition that saliva represents a pool of biomarkers. Whole saliva is a source of good diagnostic material and may serve as a substitute for blood in the monitoring, prognosis, and treatment of many diseases. Saliva contains a wide range of ingredients

that reflect the level of biomarkers in real time as well as the composition of plasma. Saliva offers many advantages, including ease and noninvasive collection, with no risk of needle injuries apart from patient fear and cooperation. Moreover, saliva compounds are characterized by a relatively long shelf life compared with blood, and easier collection procedures may provide a cost-effective approach for screening large populations and eliminate the risk of contracting infectious diseases for medical staff and patients [19].

Saliva is a biofluid with a significant number of emerging applications in research and clinical settings. It can be used as an effective diagnostic tool for systemic conditions and several medications. Unlike other common biological fluids, such as blood and urine, saliva can be easily obtained, collected, and transported for analysis [20]. Studies investigating the use of saliva as a diagnostic fluid are not limited only to the diagnosis of oral diseases; many systemic diseases, such as different types of cancer, cardiovascular disease, immunological syndromes, and hereditary disorders can also be analyzed using salivary diagnostics [21]. Screening for viral infections based on the detection of specific antibodies against viruses (e.g., hepatitis, HIV) in mucosal transudate-enriched saliva is simple and accurate. Some tumor markers (e.g., c-erbB-e, p53 antigen, CA125) present in saliva may also be used for screening and early diagnosis of malignancies that appear in other parts of the body. Similarly, oral fluids are also a source of microbial or DNA data that may be useful for biomarker profiling of oral bacteria, systemic diseases, and forensic analysis [22]. A number of caries-risk assessment tests have been developed to measure salivary bacteria, such as *Streptococci* and *Lactobacilli*. *Streptococci* are associated with an increased risk of developing caries and *Lactobacilli* are found in individuals with higher carbohydrate consumption and are also associated with an increased risk of caries [23]. Salivary tests are useful indicators of caries susceptibility at the individual level where they can be used for caries prevention and to profile for disease susceptibility. The buffering capacity of saliva is a measure of the host's ability to neutralize the reduction of plaque pH produced by acidogenic bacteria and can be measured in saliva using a commercially available kit [23]. As biomarkers are generally present at very low concentrations in the saliva, the development of specific and sensitive analytical methods is needed. Despite these limitations, the interest in saliva as a diagnostic or screening medium has advanced in recent decades [24].

Biochemistry of acute and chronic dental diseases

The oral mucosa contains an elaborate immune system and preserves immunological homeostasis and a relative state of health despite the presence of microbes [25]. The etiological link between chronic inflammatory disease and systemic conditions suggests an association between periodontal health and cardiovascular disease, DM, pre-term and low birthweight neonates, and obesity [14]. The oral cavity is constantly exposed to unique tissue-specific signals, commensal microbes and their metabolites, tissue damage from mastication, and antigens from food and airborne particles [26]. Saliva also plays an important role in the regulation of oral mucosal immune reactions and the healing of mucosal lesions, wounds, and ulcers. There are numerous defense proteins present in the saliva, such as immunoglobulins, chaperokines, cationic peptides, lysozyme, bactericidal/permeability-increasing proteins, salivary amylase, cystatins, PRPs, mucins, peroxidases, statherin, and others involved in either innate or acquired immunity. The major protective functions of salivary secretions in the oral cavity are performed through immunological and non-immunological means in addition to direct antimicrobial activity [27-30].

The major changes to connective tissue in dental disease, especially pulpitis, gingivitis, and periodontitis, are the degradation of the extracellular matrix (ECM) due to the release of enzymes by host and bacterial cells, phagocytosis of matrix components, and release of cytokines, inflammatory mediators, and apoptotic proteins. The increased levels of biomolecules and enzymes cause dental inflammation, which signifies that host-derived biomarkers cause periodontal and inflammatory diseases [31]. Periodontitis is an inflammatory disease that causes tissue damage as a result of interaction between the host immune response and pathogenic bacteria. Development of dental plaque biofilm leads to the loss of periodontal attachment and can result in tooth loss. The etiopathogenesis involves various cellular pathways, including proinflammatory mediators such as growth factors, cytokines, and MMPs. MMPs are the most important components in tissue damage associated with periodontal disease due to their role in the breakdown of ECM. The pathogens in dental plaque stimulate host cells to release MMPs, leading to tissue damage [32]. Host cell-derived enzymes such as MMPs are an important group of proteinases associated with periodontal disease. Periodontal inflammation occurs in the gingival tissue in response to bacteria biofilm. Gingivitis is characterized by an initial increase in blood flow, enhanced vascular permeability, and the influx of cells (neutrophils and monocyte-macrophages) from the peripheral blood to the gingival crevice. These cells subsequently produce cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), and immunoglobulins. The severity of periodontitis is associated with increases in IL-1β, TNF-α, prostaglandins (PE2) and MMPs, whereas their inhibition leads to a reduction in periodontal disease [33, 34]. The oral mucosa protects the host against foreign antigens and pathogenic microorganisms through an elaborate immune system composed of numerous defense and protective proteins in the saliva that are involved in oral homeostasis, immunity, and tolerance.

Laboratory diagnostics of disorders for dental treatment

The use of laboratory diagnostics has expanded enormously in the past few decades due to the development of automat-

ed analyzers and computerized systems. Treatment has improved via pathological investigations through routine tests ordered to monitor disease status, organ function, or the effect of medications. A typical laboratory report includes the patient's demographic data, date and time of specimen collection, the name of the consulting physician, and the results of pathological tests performed [35-37]. Many patients seek dental treatment when they are suffering or recovering from acute and chronic conditions or when experiencing the effects of medications. The laboratory report provides significant information regarding a patient's health that is beneficial in the diagnosis and management of oral diseases and dental treatment as well as the prognosis for a particular treatment. The information obtained from laboratory tests allows the clinician to make informed decisions regarding dental care and treatment modification to minimize complications. In addition to radiography, clinical laboratory investigations can help the dentist to diagnose oral disease, determine the severity of infection or modify medication use based on underlying diseases and proposed dental treatments [35-37].

Several diagnostic tools are available to physicians to assess patient blood glucose level. A fasting blood glucose analysis is typically used to measure the blood glucose level after an overnight fast and a postprandial sample is obtained after breakfast. The HbA1c measurement provides an estimation of the average blood glucose level over the past several months, which is used by physicians to evaluate and manage DM [38, 39]. Several complications, such as periodontal disease, salivary gland dysfunction, halitosis, burning mouth sensation, and taste dysfunction have been associated with DM. Individuals with DM are more prone to fungal and bacterial infections, oral soft tissue lesions, compromised oral wound healing processes, dental caries, and tooth loss [40]. Therefore, it is important that dentists take hematological and biochemical parameters and their potential impact on oral health and hygiene into account. Before initiating treatment for a diabetic patient, dentists must consider the laboratory analysis to minimize the risk of a diabetic emergency and reduce the probability of oral complications. A dentist should also review the patient's current DM management plan, including the dose and time of medications, as well as any lifestyle modifications, such as exercise or nutritional changes. Dentists should be mindful of their patients' health record and medication use [41].

Laboratory investigations for bleeding disorders are advisable when an individual has a personal or family history of bleeding and laboratory findings suggest the possibility of an inherited or acquired bleeding disorder. It is often advantageous to simultaneously test for von Willebrand disease, platelet function disorders, and coagulation defects, including fibrinogen disorders. An investigation for rare bleeding disorders, such as those affecting factor XIII, α2 antiplasmin and plasminogen activator inhibitor-1, is appropriate when faced with a severe congenital or acquired bleeding disorder that cannot be explained based on the results of initial diagnostic investigations. An organized strategy to investigate bleeding disorders considers the important issues, confirms abnormal findings, fosters proper interpretation of the results, and provides a helpful framework for assessing the cause of bleeding [42].

Conclusion

A thorough understanding of modern biochemistry and molecular biology will be useful to students of medicine, including but not limited to dentistry, and would allow greater applications of research and teaching. Hence, an important aspect in biochemistry instruction is an emphasis on the fact that the basic biochemical processes occurring in the oral cavity are the same as those taking place in other tissues and organs of the body. Saliva is secreted from the salivary glands and has multiple functions, including mouth cleansing and protection, antibacterial activity, and assisting digestion. With the rapid advancement in salivaomics, saliva is now well recognized as a source of biological markers, including DNA, RNA and proteins, and those in microbial biofilm. The major changes that occur in the connective tissue in cases of dental diseases, particularly pulpitis, gingivitis, and periodontitis, are the degradation of extracellular matrix due to the release of enzymes by host and bacterial cells, phagocytosis of matrix components and the release of cytokines, inflammatory mediators, and apoptotic proteins. The elevated levels of these molecules and enzymes reflect the degree of dental inflammation, indicating that host cell-derived molecules can lead to the development of periodontal and inflammatory diseases. Given the numerous possible oral manifestations of DM and the risk of a diabetic emergency, it is important for dentists to recognize and appreciate the impact of this disorder on dental care. A thorough understanding of DM and the implications for dental management are necessary for a dentist to provide optimal oral health care to diabetic patients. Testing for bleeding disorders is advisable when a patient presents with a personal or family history of bleeding and laboratory investigations suggest there may be an inherited or acquired bleeding disorder. An advanced laboratory investigation is helpful in the diagnosis of various blood disorders, including those associated with coagulation factors, clot stabilization, or fibrinolysis. Clinical and biomedical research has contributed greatly to advancements in dental and medical sciences. Medical and clinical biochemistry is becoming increasingly significant in dentistry, and should be recognized in both teaching and research.

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