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Establishment of Reference Interval for M2BPGI Glyco-Biomarker in the Serum of Mongolian People

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Objectives: Establishment of reference interval for M2BPGI glyco-biomarker in the serum of relatively healthy Mongolian people. **Methods:** Serum analysis for M2BPGI glyco-biomarker, hepatitis virus markers, and AFP analysis were performed using a fully automated chemiluminescence immunoassay analyzer. Non-parametric methods were used for establishment of reference interval according to the guideline of the Clinical Laboratory Standards Institute. Statistical analysis was done by using SPSS ver. 20.0; SPSS Inc., Chicago, IL software. **Results:** Total of 3384 people were involved in this study. Of the total participants, 40% were overweight and 31.3% were obese. Also, of the population 10.1 percent were positive for HBsAg, 17 percent were positive for Anti-HCV, and 1.1 percent had co infection of both HBV and HCV. A total of the 316 individuals met the inclusion criteria for reference interval establishment and 77 (24.4%) of them were men and 239 (75.6%) were women. The reference interval of M2BPGI glyco-biomarker for men was 0.883 ± 0.311C.O.I and for women was 0.951 ± 0.411C.O.I. **Conclusion:** It is determined that the reference interval for M2BPGI glyco-biomarker in the serum of relatively healthy Mongolian people was 0.936 ± 0.391C.O.I.

Keywords: Mac-2, Biomarkers, Binding Protein, Serum, Mongolia

Introduction

Approximately 80% of the secretory proteins found in body fluids as well as cell membrane proteins are N-glycosylated with specific asparagine residues and are present as glycoproteins [1]. Furthermore, most serum glycoproteins, except immunoglobulins, are secreted from the liver. Therefore, the quantification of the qualitative glycan structural change on the target glycoprotein and establishment of a simple and reliable assay system is the key to the development of a new glyco-biomarker for disease diagnosis [2, 3].

Hepatic fibrosis is the result of the wound-healing response of the liver to repeated injury, and it affects the entire liver tissue [4]. When the acute hepatitis progresses to chronic hepatitis, the liver becomes fibrotic and the stiffness of the liver proceeds toward cirrhosis. These changes occur over 10-15 years and may present as a risk of hepatocellular carcinoma in the age of over 40. Consequently, the glycan structures of many serum glycoproteins are affected due to the progress of the disease, thus, detection of the glycoproteins with altered glycosylation should be relatively easy, which would result in excellent detection sensitivity.

The serum diagnostic agent for liver fibrosis, Mac-2 binding protein with specific glycosylation 'M2BPGI assay kit' was recently developed and went through efficacy evaluation with more than 8000 clinical samples [5]. The M2BPGI assay kit was approved as an efficient diagnostic agent, and the diagnosis expense is supported by the Japanese public health insurance as of January 2015. In Mongolia, M2BPGI glyco-biomarker analysis was introduced in 2015 and clinical laboratories are using it for clinical and research purposes.

In the M2BPGI glyco-biomarker analysis, the reference values are usually defined based on clinical test values that can be obtained from healthy subjects. These values are the basic yardstick for clinical interpretation of test results. Therefore, the concept of reference intervals is not set for comparison against disease, but groups of healthy subjects, and is the result of tests that should be found in healthy subjects. Ideally, reference intervals should be treated as universal, unaffected by region, era, subjects, etc., but in practice, it may be necessary to set reference intervals separately for each region, or for each purpose for which they are used.

In the past, the expression "normal value" was also used, but considering the adverse effect that those within the reference interval could be interpreted as normal (healthy) and those outside the range as abnormal (sick), the term reference interval has been used recently [6-9]. Since no studies have been conducted on the establishment of the reference interval values for M2BPGI glycol-biomarker from the time it was introduced in Mongolia, we aimed to determine these values specifically for Mongolian people in this study.

Materials and Methods

Sampling

This study was conducted according to the guidelines and standards of the Clinical and Laboratory Standards Institute (CLSI, formerly called the NCCLS (National Committee for Clinical Laboratory Standards)) and C28-A3, 2008.

For the selection of the Reference population, we have considered the administrative and geographical features of Mongolia, and sampling was conducted at three levels: the capital city, provincial centers, and rural areas. They are specifically: the population of Bayanzurh district, Sukhbaatar district, and Chingeltei district of the capital city of Ulaanbaatar city; Gobi-Altai province and Uvs province in the western region; Arkhangai and Khuvsgul provinces in the khangai region; Tuv, Dornogobi, and Umnugobi province in central the region; and lastly, Sukhbaatar province in the eastern region. Totally 3384 participants were selected. The sample size was estimated using a multi-stage random sampling method (simple random, well-organized, and cluster sampling) from October 2016 to January 2019. Stated statistically:

$$n = DE * \frac{Z^2 * p * (1-p) * 1.2}{e^2} = 1.5 * \frac{1.96^2 * 0.10 * (1-0.10) * 1.2}{0.014^2} = 3175$$

Where parameters: n= sample size, p=expected prevalence, Z=statistic for a level of confidence, e=the acceptable sampling error, and D=desired margin of error. The reference sample group was selected from the reference population by excluding people who do not meet the inclusion criteria as specified in the following inclusion and exclusion criteria. According to the guidelines of the Clinical and Laboratory Standards Institute, a minimum of 120 specimens can be used, however, considering the abnormal distribution due to the abnormal results, as many specimens as possible, or at least 300 specimens should be used.

Inclusion criteria

As citizens of Mongolia, both the participant, and the caregiver must have approved to participate in the study.

Exclusion criteria

By questionnaire for current illness such as consumption of alcoholic drinks and cigarettes, liver disorders, and regular medicines or drugs used during last one month as well as diabetes.

By measurement of body for overweight and obesity evaluated by waist circumference of more than 90 cm for men and 80 cm for women.

By abnormal laboratory tests including HBsAg positive, anti-HCV positive, increased level of AFP more than 10 IU/ml, and increased AST or ALT level.

Laboratory testing

We evaluated qHBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc to detect and prove hepatitis B virus infection, and Anti-HCV to detect hepatitis C virus. Non-invasive liver fibrosis marker M2BPGI was determined by using chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol for the fully automated immunologic analyzer HISCL-5000 of the Sysmex Corporation in Japan. The sensitivity of this method is 100% and specificity is 99.94%. To evaluate liver function, the ALT and AST levels were evaluated using the kinase-UV method recommended by the International Clinical Chemistry Association (IFCC) and the Chemix-180 (Sysmex, Japan), Japanese Clinical Chemistry Association (JSCC) and measured by a biochemical full-automatic analyzer.

Statistical analysis

Descriptive statistics and frequency distributions were computed for all the variables. The data were tested for normality using the Shapiro-Wilk test. For continuous variables, the independent t-test was used for comparing two groups. One-way ANOVA was carried out for more than two groups. Multiple comparison tests were not performed because all groups were not significantly different from ANOVA. The Chi-square test was used for categorical data. Statistical significance was determined at a p value lower than 0.05. All statistical analysis was performed using SPSS (version 25).

Ethical statement

The research study was approved by the Research Ethics Committee of the Mongolian National University of Medical Sciences (№8/3/2016-08). All participants gave written informed consent.

Results

Baseline characteristics of participants

A total of 3384 people under the age of 40-65 were randomly selected, and the statistical data was processed for 3196 respondents who completed all stages of the study. Of the total respondents, 1093 (34.2%) were male and 2103 (65.8%) were female. Of the total participants in this study, 1829 (57.2%) were from Ulaanbaatar, while 1367 (42.8%) of the total participants were living in the 8 selected provinces (Table 1).

Table 1. Demographics of reference population.

Variables	N	%
Area		
Arhkhangai	270	8.5
Govi-Altai	177	5.5
Dornogovi	135	4.2
Sukhbaatar	206	6.4
Umnugovi	94	3.0
Tuv	154	4.8
Uvs	199	6.2
Khuvsgul	132	4.2
Ulaanbaatar	1829	57.2
Location		
Rural	1367	42.8
Urban	1829	57.2
Gender		
Male	1093	34.2
Female	2103	65.8
Age group		
40-44	958	30
45-49	758	23.7
50-54	677	21.2
55-59	522	16.3
60-64	281	8.8

Table 2. Body Mass Index: age, sex, region difference.

Variables	Underweight	Normal weight	Overweight	Obese	p-value
Age	**N (%)**	**N (%)**	**N (%)**	**N (%)**	
40-44	14 (1.4)	279 (28.2)	417 (42.1)	280 (28.3)	0.000
45-49	4 (0.5)	253 (32.2)	304 (38.7)	225 (28.6)	
50-54	4 (0.6)	184 (26.2)	290 (41.3)	224 (31.9)	
55-59	4 (0.7)	135 (24.7)	217 (39.7)	191 (34.9)	
60-64	7 (2.4)	67 (23.1)	98 (33.8)	118 (40.7)	
Sex					
Male	12 (1.1)	349 (30.6)	445 (39.0)	335 (29.4)	0.049
Female	21 (1.0)	569 (26.2)	881 (40.5)	703 (32.3)	
Region					
Rural	11 (0.8)	390 (28.7)	528 (38.8)	431 (31.7)	0.472
Urban	22 (1.1)	528 (27.0)	798 (40.8)	607 (31.0)	

p-value has been calculated with Chi-square test

As to prevalence of Hepatitis B and C virus infection, of all participants, 71.8 percent were negative for virus markers, 10.1 percent were positive for HBsAg, 17 percent were positive for Anti-HCV, and 1.1 percent had co-infection of both HBV and HCV. According to gender, there were 71.9% of males and 71.8% of women with a negative result on viral markers, 14.8% of males and 7.7% of women were HBsAg positive. 12.8% of men and 19.2% of women were anti-HCV positive. 0.46% of men and 0.39% of women were both HBsAg and anti-HCV positive. Results were statistically significantly different ($p < 0.000$) (Table 3).

Table 3. Comparison of HBsAg, Anti-HCVmarker result by gender, age and location.

Variables	No Virus Hepatitis Infections		HBsAg positive		Anti-HCV positive		HBsAg+Anti-HCV positive		
	N	% (95% CI)	N	% (95% CI)	N	% (95% CI)	N	% (95% CI)	p-value
Gender									
Male	786	71.9 (69.2-74.5)	162	14.8 (12.8-17.0)	140	12.8 (10.9-14.9)	5	0.46 (0.17-1.00)	0.000
Female	1509	71.8 (69.8-73.6)	162	7.7 (6.6-8.9)	403	19.2 (17.5-20.9)	29	1.38 (0.94-1.95)	
Age group									
40-44	734	76.6 (73.9-79.2)	110	11.5 (9.6-13.6)	108	11.3 (9.4-13.4)	6	0.63 (0.26-1.29)	0.000
45-49	575	75.9 (72.7-78.8)	76	10.0 (8.0-12.3)	101	13.3 (11.0-15.9)	6	0.79 (0.33-1.62)	
50-54	456	67.4 (63.8-70.8)	70	10.3 (8.2-12.8)	143	21.1 (18.2-24.3)	8	1.18 (0.56-2.22)	
55-59	351	67.2 (63.1-71.2)	46	8.8 (6.6-11.5)	118	22.6 (19.2-26.3)	7	1.34 (0.60-2.61)	
60-64	179	63.7 (58.0-69.2)	22	7.8 (5.1-11.4)	73	26.0 (21.1-31.3)	7	2.49 (1.12-4.83)	

HBsAg -Quantitative hepatitis B surface antigen; Anti-HCV - Antibodies against hepatitis C virus; p-value has been calculated with Chi-square test

AFP protein, a liver cancer marker, was increased in 50 people. Of these, AFP protein levels were elevated in 16 men and 34 women, and there was no statistically significant difference ($p = 0.488$). AFP protein levels were elevated in 23 people from Ulaanbaatar and 27 people from rural areas (Table 4).

Table 4. Number of people with increased AFP protein above 10IU/ml.

Variables	AFP increased			
	All	N	% (95%CI)	p-value
Region				
Rural	1363	27	2.0 (1.3-2.8)	0.301
Urban	884	23	2.6 (1.7-3.9)	
Sex				
Male	830	16	1.9 (1.2-3.1)	0.488
Female	1417	34	2.4 (1.7-3.3)	

Results of serum analysis for M2BPGI glyco-biomarker reference interval of Mongolian people

The level of M2BPGI glyco-biomarker was determined in the serum of a relatively healthy population of Mongolia as a reference interval. A total of 3384 people was randomly selected in this study and 316 of them met the inclusion criteria for the analysis of M2BPGI glyco - biomarker. It is determined that the reference interval of M2BPGI glyco - biomarker was 0.936 ± 0.391C.O.I .

Of the 316 individuals who met the inclusion criteria for reference interval establishment, 77 (24.4%) were men and 239 (75.6%) were women. The reference interval of M2BPGI glyco-biomarker for men was 0.883 ± 0.311C.O.I and for women was 0.951 ± 0.411C.O.I. The average value of the reference age groups fluctuated between 0.921 and 0.997C.O.I (Table 5).

Table 5. M2BPGI reference range by age and gender, in C.O.I.

	Variables	N	Average	Standard dev	Geometrical average	Median	[a]p-value
Sex	Male	77	0.883	0.311	0.834	0.815	0.512
	Female	239	0.951	0.411	0.875	0.830	
Age	40-44	110	0.922	0.393	0.851	0.810	0.841
	45-49	94	0.926	0.401	0.857	0.800	
	50-54	59	0.921	0.394	0.847	0.840	
	55-59	37	0.997	0.391	0.931	0.905	
	60-64	16	0.997	0.343	0.939	1.075	

M2BPGI– Mac-2-binding protein biomarker; [a]p-value has been calculated with Chi-square test

The results of serum M2BPGI glyco-biomarker analysis in the total participants in the study of liver fibrosis

Serum M2BPGI glyco-biomarker analysis has been done in 3172 people to detect liver fibrosis and an increased level of M2BPGI has been recorded in 46.8% of the participants. The frequency of increased level of M2BPGI is seen more in older age groups, specifically, it was 39.7% of the participants age 40-44, 40.1% in age 45-49, 51.9% in age 50-54, 54.5% in age 55-59, and 62% in age group 60-64. In 42.9% of the men and 48.9% of women the level of M2BPGI glyco-biomarker was increased ($p < 0.001$). When comparing urban and rural cases of high incidence of M2BPGI glyco-biomarkers, it was 43.9% in rural areas (95.0% presentation interval: 40.8% -46.0%) and 49.4% in the urban areas (95.0% presentation interval: 47.1% -51.7%) ($p < 0.001$) (Table 6).

Table 6. The increased level of M2BPGI (urban and rural, age, sex).

Variables	M2BPGI Positive			
	All	N	% (95%CI)	p-value
Region				
Rural	1366	592	43.4 (40.8-46.0)	0.001
Urban	1806	892	49.4 (47.1-51.7)	
Gender				
Male	1077	460	42.7 (39.7-45.6)	0.001
Female	2095	1024	48.9 (46.8-51.1)	
Age group				
40-44	947	376	39.7 (36.7-42.9)	0.000
45-49	753	302	40.1 (36.6-43.6)	
50-54	676	351	51.9 (48.2-55.7)	
55-59	512	282	54.5 (50.2-58.8)	
60-64	279	173	62.0 (56.2-67.6)	

M2BPGI– Mac-2-binding protein biomarker; ap-value has been calculated with Chi-square test

Serum M2BPGI glyco-biomarker was negative in 60.6% of people with normal weight, 35.1% (+), 4.3% (++), 51.6% in overweight people, 45.4% (+) (++) in 3.0% (++), 45.3% in obese people, 50.6% in (+), 4.0% (++), indicating higher the body mass index higher the M2BPGIglyco-biomarker ($p < 0.000$). There was a statistically significant difference between BMI, age group, and sex ($p < 0.000$) (Table 7).

Table 7. M2BPGI change: age, sex, region.

M2BPGI	COI< 1.0 (negative)	1.0<COI< 3.0 (+)	COI >3.0 (++)	
	N (%)	N (%)	N (%)	p-value
BMI				
Normal	524 (32.6)	303 (22.3)	37 (32.4)	0.000
Overweight	643 (40.1)	566 (41.7)	38 (33.3)	
Obese	438 (27.3)	489 (36.0)	39 (34.2)	
Age				
40-44	567 (34.1)	360 (25.9)	18 (15.5)	0.000
45-49	444 (26.7)	287 (20.7)	21 (18.1)	
50-54	321 (19.3)	318 (22.9)	35 (30.2)	
55-59	228 (13.7)	267 (19.3)	22 (18.9)	
60-64	105 (6.3)	154 (11.1)	20 (17.2)	
Sex				
Male	613 (36.8)	425 (30.6)	39 (33.6)	0.002
Female	1052 (63.2)	961 (69.3)	77 (66.3)	
Region				0.001
Rural	767 (46.1)	559 (40.3)	39 (33.6)	
Urban	898 (53.9)	827 (59.6)	77 (66.3)	

M2BPGI– Mac-2-binding protein biomarker; [a]p-value has been calculated with Chi-square test

When comparing M2BPGI glyco-biomarkers with a change in body weight in non-infected and infected hepatitis virus groups, the M2BPGI glyco-marker is increased with increasing body mass. This was an interesting result, as it was statistically significantly higher in the non-hepatitis group ($p < 0.000$), while the change in body weight of the coinfection of hepatitis B and C virus-infected group was no statistically significant difference (Table 8).

Table 8. M2BPGI glyco-biomarker analysis in the groups with body weight changes and the results shown by non-infected and infected hepatitis virus groups.

Variables	Non-infected group		Infected group with HBV		Infected group with HCV		Infected group with HCV+HBV		*p-value
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	
Low weight	24	0.74 ± 0.29	3	0.62 ± 0.25	5	1.17 ± 0.90	-		
Normal weight	590	0.92 ±0.63	108	1.68 ± 1.37	169	1.65 ± 1.67	13	1.71 ± 0.82	0.229
Overweight	909	1.01 ± 0.45	115	1.46 ± 1.20	211	1.78 ± 1.47	14	2.17 ± 1.39	0.073
Obesity	724	1.07 ± 0.59	92	1.85 ± 1.97	152	2.15 ± 2.32	7	1.54 ± 0.97	0.397

*One-way ANOVA result.

Discussion

Establishing a reference range value of newly introduced laboratory analysis in the clinical experience of the country is a key criterion for clinical interpretation and it is required to be set in a regional or for a specific purposes [9]. In Mongolia, non-invasive M2BPGI glyco-biomarker analysis was introduced in 2015 and used for clinical and research purposes by clinical laboratories. Since no studies have been conducted on the establishment of the reference interval for M2BPGI protein since it was introduced in Mongolia, we aimed to determine these values specifically for Mongolian people in this study.

According to the guidelines and standards of the Clinical and Laboratory Standards Institute, a total of 3384 participants were randomly selected for the reference population of this study, and the proportional ratio is considered for demographic indicators of age and gender based on the Population Demographics Statistics of Mongolia for 2015. Body measurements and laboratory tests were performed on 3196 participants after alcohol consumers were excluded. For the reference sample group, it is important to select relatively healthy people, who meet the inclusion criteria for the establishment of reference interval values. Therefore, we have examined the factors for liver fibrosis, such as the use of medication, overweight and obesity, hepatitis B and C infections, and liver cancer among the participants.

According to the guidelines of the Clinical and Laboratory Standards Institute, a minimum of 120 specimens can be used, however, considering the abnormal distribution due to the abnormal results, as many specimens as possible, or at least 300 specimens should be used. The level of M2BPGI glyco-biomarker was determined in the serum of 316 people, who met the inclusion criteria for the analysis of the M2BPGI glyco-biomarker. It is determined that the reference interval value of the M2BPGI glyco-biomarker was 0.936 ± 0.391C.O.I. In Japan, the M2BPGI glyco - biomarker was COI< 1.0, which was determined by the clinical trial study [8].

Fatty liver is classified as alcoholic or non-alcoholic, and non-alcoholic fatty liver disease is caused by obesity. The study found that about 90% of obese people suffer from fatty liver disease [10-12]. Patients with NASH have a 20%–50% risk of developing progressive inflammation or liver fibrosis and have a 2%–20% 5-year cumulative incidence of hepatocellular carcinoma [13-15]. According to a joint study, conducted by the WHO, the Millennium Challenge Foundation and the Public Health Institute, in 2009, 42.7% of the population aged 15-64 years were overweight or obese. We found that 40% of our participants were overweight and 31.3% were obese. This is because the age range of our study subjects was between 40 and 64 years old. In a clinical trial study, the comparison is made between M2BPGI glyco-biomarker and liver biopsy and they are well matched. Specifically, the M2BPGI was negative in the group without chronic hepatitis, and 1.0 <COI<3.0

/ + / in the group with chronic liver diseases, and COI> 3.0 / ++ in the liver cirrhosis group. The results of our study have shown that M2BPGI was negative in 51.6% of overweighed people, 45.4% is (+), 3.0% is (++), 45.3% of obese people are negative, 50.6 % (+), 4.0 % (++). These findings are similar to the results of other researchers who have shown that hepatic inflammation and fibrosis changes are recorded in 20-50% of obese individuals. In the studies, conducted by Abe M, Miyake T, Kuno A, et al. M2BPGI glyco-biomarker was found to be an effective glyco-biomarker for the evaluation of liver fibrosis in patients with alcoholic and non-alcoholic fatty liver disease [16, 17]. A joint study was conducted in which medical doctors from 15 hospitals in Japan were involved with more than 8,000 specimens collected from patients diagnosed with liver fibrosis (F0-F4). In the joint study, liver tissue tests confirm that M2BPGI is a clinically useful glyco-biomarker. M2BPGI is highly effective in diagnosing the degree of fibrosis in a person infected with the hepatitis C virus [18], and high levels of M2BPGI may be associated with increased risk of hepatocellular carcinoma [19].

The diagnostic capabilities of M2BPGI marker are not only similar to the results obtained from the tissue strain analytics as in reference, ultrasound Solution for Elastography (Siemens, Mountain View, CA, USA, but also more effective than the other selective markers. The analysis of 707 patients with hepatic C virus showed that increasing M2BPGI levels was positively proportional to increased risk of hepatocellular carcinoma, and that patients with M2BPGI levels 4.2 and higher were at higher risk for hepatocellular carcinoma. The researchers concluded that M2BPGI was effective in assessing the degree of fibrosis of patients with hepatitis B and C. In our study, we found that in the patients with hepatitis C virus and concomitant infections, M2BPGI protein levels were higher when compared with the patients with hepatitis B virus. And, in non-infected people, obesity is also a risk for liver fibrosis ($p < 0.000$) [20-23].

We acknowledged that there are several limitations for the current study. First, however, the study has only a relatively small sample size. Only 316 (8.2%) individuals met inclusion criteria for reference interval establishment. Second, as the subjects were all Mongolian, the generalizability of any finding in this study to other ethnicities remains to be evaluated. Further studies are required to assess serum M2BPGI level as a serum biomarker in extensive patient populations, including non-Asian patients, especially those with severe liver fibrosis.

Conclusion

The reference range for M2BPGI glyco-biomarker in the serum of relatively healthy Mongolian people age 40-65, was 0.936 ± 0.391C.O.I. High levels of M2BPGI protein were recorded in persons with co-infection of hepatitis B virus, hepatitis C virus, and other co-infection. Elevated M2BPGI glyco-biomarke indicate risk of liver fibrosis and cancer. Changes in body weight in people without the hepatitis B virus can also lead to liver fibrosis.

Conflict of Interest

The authors declare that they have no competing interests.

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