Clinical Biochemistry
Research Article – 27003

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Comparison of classical and clustering based reference interval calculations for Aspartate Aminotransferase, Alanine Aminotransferase and Gamma-Glutamyl Transferase in serum

Aspartat Aminotransferaz, Alanin Aminotransferaz ve Gama Glutamil Transferaz serum için klasik ve kümelemeye dayalı referans aralık hesaplamalarının karşılaştırılması

Abstract: Objective: There are fixed rules applied to determine the reference intervals (RIs) of the biochemical tests. However, these rules lack for identifying subgroups within the reference population. Therefore, we suggest the clustering method, which determines the sub-groups by taking the correlations between the variables into account in the RIs calculations. In our study, it is aimed to compare the results of the RIs based on the clusters analysis with the results of the conventional method.

Methods: The individuals who applied Ankara Düzen Laboratory for the check-up with normal Ultra Sono Grafi (USG) in 2012–2014 and who have had Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and G-Glutamyl Transferase (GGT); (U/L) results were included in this study. We have excluded the repeated applies of patients, only analyzed the first apply to the laboratory. Reference individuals are composed of 883 people. (610 male, 273 female, 18–70 years). Non-parametric methods were used to determine reference intervals. Fuzzy C-Means clustering method was used to identify sub-groups.

Results: AST, ALT, GGT measurements for all of the check-up individuals were determined by non-parametric method for the three subgroups specified after the Fuzzy-C-Means clustering method and the entire group. According to the reference intervals obtained, the third sub-cluster derived from the group intended to be used as the reference population was observed as a cluster that is narrower, and has similar properties of the actual reference population. However, when the correlations between the tests in the sub-groups are considered, the correlations between GGT-ALT-AST have been observed to be higher while the correlation level between ALT-AST in the group proposed as a real reference population does not change.

Conclusion: In the reference limit studies, instead of the determination of the reference interval for a single group designated as the reference population, we think that, the subgroups which are homogeneous within itself, heterogeneous between themselves should be set in in this group. In determining multiple sub-groups, the relationship between more than one test need to be taken into consideration, and the effect of clustering should be investigated.

Keywords: Reference interval, liver function tests, fuzzy-C means, data mining

Özet: Amaç: Biyokimyasal testlerin referans aralıklarının belirlenmesi için kullanılan sabit kurallar bulunmaktadır. Ancak bu kuralların referans popülasyon içerisindeki alt grupların tanımlanmasında eksikleri bulunmaktadır. Bu nedenle RIs hesaplamasında değişkenler arasındaki korelasyonları göz önüne alarak alt grupları belirleyen kümeleme yöntemini önermekteyiz. Çalışmamızda kümele analizine dayalı referans aralıklar ile klasik yöntemin sonuçlarının karşılaştırılması amaçlanmıştır.

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Metod: Referans bireyler 883 kişiden oluşmaktadır. (610 erkek, 273 kadın, 18–70 yaş). Referans aralıkların belirlenmesinde parametric olamayan yöntemler kullanılmıştır. Alt grupların belirlenmesi için Fuzz-C-means kümeleme yöntemi kullanılmıştır.

Bulgular: Check-up bireylerin tamamında AST, ALT, GGT ölçümleri Fuzzy-C-Means kümeleme yöntemi sonrası belirlenen üç alt grup için ve tüm grup için parametrik olmayan yöntem ile belirlenmiştir. Elde edilen referans aralıklara göre referans popülasyon olarak kullanılması planlanan gruptan elde edilen 3. alt kümenin daha dar ve gerçek referans popülasyon özelliklerine sahip olduğu gözlenmiştir.

Sonuç: Referans limit belirleme çalışmalarında referans popülasyon olarak belirtilen tek bir grup için referans aralıkların belirlenmesi yerine, bu grup içerisindeki kendi içinde homojen, birbirleri arasında heterojen alt grupların belirlenmesi gerektiğini düşünmekteyiz. Bu alt grupların belirlenmesinde birden fazla test arasında ilişkilerin de göz önüne alınması ve etkisinin araştırılması gerekmektedir.

Anahtar Kelimeler: Referans aralık, karaciğer fonksiyon testi, bulanık C-ortalamalar kümeleme, veri madenciliği

DOI 10.1515/tjb-2015-0030
Received May 4, 2015; accepted July 11, 2015

Introduction

Some concepts are necessary to be known very well before starting the reference interval studies. These concepts are; the reference individual, the reference population, the reference samples, the reference values and the limit (Figure 1). The reference individual is a person selected according to well-defined criteria [1]. These are the inclusion criteria and exclusion criteria. The reference population defines the mass consisted of the individuals that have the characteristics (clearly defined) aimed to be investigated in the study [2]. The number of people in the reference population is not known. Therefore, it is a theoretical expression. It can even be a single person if the person is used as a reference of himself [3–15]. Reference sample is used for the group formed from the reference population by considering certain criteria. There are two methods for the reference sample selection: 1) Indirect Sampling, 2) Direct Sampling. While in Indirect Sampling the individuals are selected from a ready data set according to the criteria,

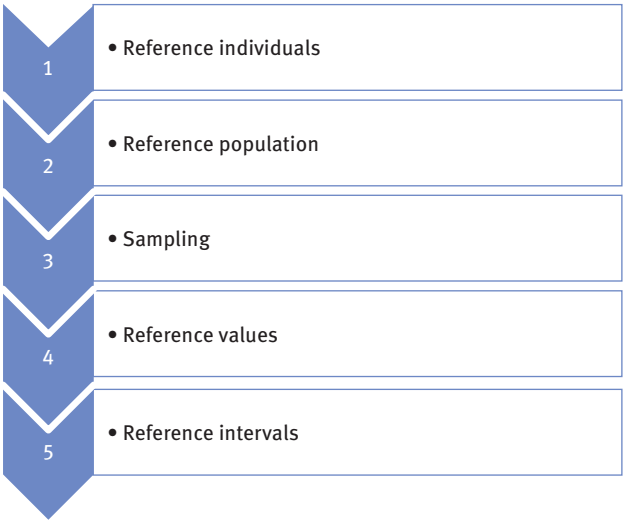

Figure 1: Component of determination of reference interval.

in the Direct Sampling the criteria are used when selecting the individuals [6]. Reference interval is the interval in which the reference values obtained by using certain statistical analysis are defined in the sample reference distribution [4]. Reference value and interval are used for the interpretation of the tests used in clinical biochemistry laboratories. The test results of different laboratories are seriously affected by various factors (E.g. age, gender).The need to carry out work on considering what values and how to evaluate tests for the individuals that have different status has emerged in recent years. With the help of the reference values and limits, the decision makers are able to distinguish individuals as sick or healthy.

In this study, we have evaluated the reference intervals for ALT, AST, GGT in two ways:

1) Classical reference interval determination techniques -the non-parametric

2) Identifying the subgroups with Fuzzy C-Means clustering method and the determination of the reference intervals afterwards (Figure 2).

The reason of selecting these tests is that they are very important in Liver function screening, diagnosis, treatment. Therefore, the calculations of RI become important

Our purpose is also to indicate the existence of dissimilar subgroups in the groups considered as the reference individuals. It is comparatively shown that the reference intervals for this subgroups should be determined separately. With this study, each hospital or laboratory can evaluate their reference intervals for its own population more accurately by performing the subgroup analysis [1–3].

In one of the multicenter reference interval studies for these three tests, the researchers compared the data from Italy, Turkey and China [16]. They specified the ref-

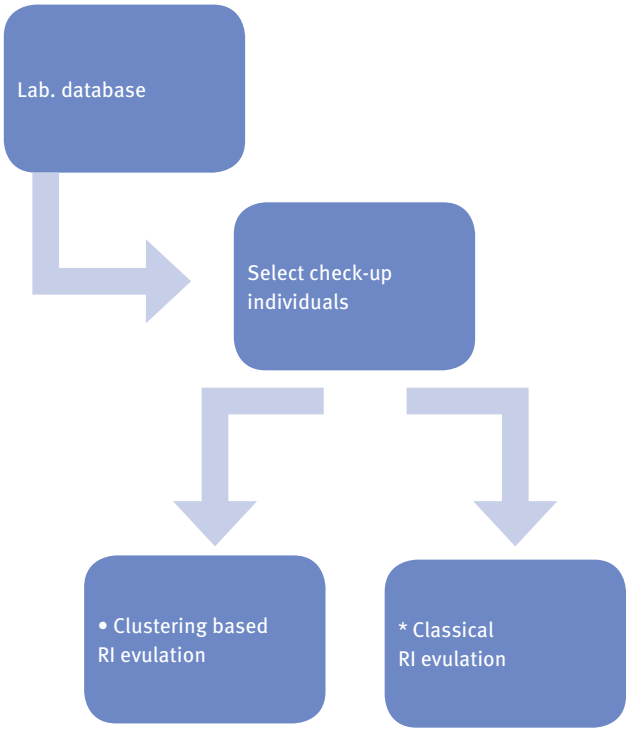

Figure 2: Pipeline of analysis.

erence intervals for the male and female, and defined the differences by regions. They have mentioned that AST results from the four regions (Milan, Beijing, Bursa and Nordic Countries) were statistically different, but these differences were too small to be clinically relevant. They have reported that interregional differences were not statistically significant for ALT but partitioning was required due to significant gender differences. The upper reference intervals for GGT from the Nordic Country population were higher than those from the other three regions and results from this group were excluded from final calculations. They have also reported that the question why these differences occur should be investigated in more detail. In another study, the researchers measured ALP, total protein, albumin and total bilirubin values for the Chinese adult population, in addition to the three tests within the study [17]. They mentioned that they have established common reference intervals of liver function tests that are defined specifically for Chinese population and can be universally used among EQA-approved laboratories located all over China [17]. As seen in other studies in the literature, RI studies of Liver function tests are clinically very important. The purpose of our study is to put forth how a bias is introduced by ignoring the hidden groups that could be ignored without analyzing rather than redefining the reference intervals.

Materials and Methods

Fuzzy c-means

The FCM algorithm is a widely used technique that uses the principles of fuzzy sets to evolve a partition matrix U(X) while minimizing the measure [14,15],

$$J_m = \sum_{j=1}^{n}\sum_{k=1}^{K} u_{k,j}^{m} D^2(z_k, x_j), \quad 1 \le m \le \infty$$

where n is the number of data objects, K represents number of clusters, $u_{k,j}$ is cluster membership of jth point in the kth cluster and m denotes the fuzzy exponent. $D(z_{k,\,xj})$ denotes the distance of point x_j from the kth cluster center z_k. FCM algorithm starts with random initial K cluster centers, and then at every iteration, it finds the fuzzy membership of each data points using the following equation [15]:

$$u_{k,i} = \frac{\left(\frac{1}{D(z_k, x_i)}\right)^{\frac{1}{m-1}}}{\sum_{j=1}^{K}\left(\frac{1}{D(z_j, x_i)}\right)^{\frac{1}{m-1}}}, \quad \text{for} \quad 1 \le k \le K, \quad 1 \le i \le n$$

The cluster centers are recomputed using the following equation:

$$z_k = \frac{\sum_{i=1}^{n} u_{k,i}^{m} x_i}{\sum_{i=1}^{n} u_{k,i}^{m}} \quad 1 \le k \le K$$

The algorithm terminates when there is no further change in the cluster centers. Finally, each data point is assigned to the cluster to which it has maximum membership [15]. In this study, the reason of preferring this method as the clustering method is that it gives more compact results than the conventionally used k-means, k-medoids or hierarchical clustering algorithms [18,19].

Table 1: Methods of tests.

Test	Method
AST	Kinetic assay, according to IFCC without pyridoxal phosphate activation
ALT	Kinetic assay, according to IFCC without pyridoxal phosphate activation
GGT	Enzymatic colorimetric assay, standardized against International Federation of Clinical Chemistry (IFCC)

Table 2: Tests %CV values, and 2 level internal control sample information.

Test	PCCM-1	PCCM-2
	N=120 (60* 2)	
	CV (%)	CV (%)
AST	1.83	1.70
ALT	2.54	1.73
GGT	2.95	2.31

Reference population

Reference population includes 883 individuals who applied Düzen laboratories, which is one of the largest laboratories in Ankara, the capital of Turkey, for a check-up in 2012–2014, and whose USG results were normal. 610 of these individuals are male and 273 are female. We have excluded the repeated application of patients, just have analyzed the first application to the laboratory. No personal information of the individuals was used for this analysis. IT staff of laboratory have extracted and send the data anonymously for all records.

Biochemical analytes and analytical system

Analytical measurements have been studied on the Cobas c 501 the auto analyzer produced by 'Roche Diagnostics GmbH' using the reagents that were produced for this device by the same manufacturer. The methods used are presented in Table 1. Test calibrations were performed by Calibrator for Automated System (CFAS) calibrator which was produced by the manufacturer.

The test parameters were routinely checked once a

Table 3: Descriptive statistics of tests according to the gender for all data and subsets.

			Cluster			All data
			1	2	3	
GGT (U/L)	Male	Mean	22.94	52.70	51.46	32.08
		Standard deviation	7.82	23.00	23.07	19.73
		Median	22.00	47.00	45.00	26.00
		Minimum	8.00	18.00	17.00	8.00
		Maximum	61.00	122.00	127.00	127.00
	Female	Mean	15.62	40.00	41.21	17.55
		Standard deviation	7.07	17.24	16.49	10.53
		Median	14.00	40.00	42.50	14.00
		Minimum	6.00	14.00	20.00	6.00
		Maximum	46.00	67.00	67.00	67.00
AST (U/L)	Male	Mean	20.55	35.74	23.94	22.73
		Standard deviation	4.48	10.93	5.34	7.10
		Median	20.00	34.00	23.00	21.00
		Minimum	11.00	19.00	15.00	11.00
		Maximum	42.00	67.00	45.00	67.00
	Female	Mean	18.25	48.29	28.57	19.55
		Standard deviation	4.17	13.59	9.36	7.16
		Median	18.00	46.00	29.00	18.00
		Minimum	11.00	27.00	15.00	11.00
		Maximum	36.00	69.00	50.00	69.00
ALT (U/L)	Male	Mean	24.86	71.00	35.77	31.60
		Standard deviation	8.52	23.37	10.26	17.42
		Median	24.00	65.00	35.00	27.00
		Minimum	10.00	45.00	14.00	10.00
		Maximum	50.00	179.00	77.00	179.00
	Female	Mean	17.60	61.14	32.00	19.45
		Standard deviation	6.91	14.55	14.91	10.74
		Median	16.00	56.00	32.50	17.00
		Minimum	6.00	46.00	13.00	6.00
		Maximum	49.00	82.00	72.00	82.00

Table 4: The distribution of individuals in the clusters according to the gender.

	C1		C2		C3	
	#	%	#	%	#	%
Male	85	50.3	136	68.0	389	75.7
Female	84	49.7	64	32.0	125	24.3
Total	169	100.0	200	100.0	514	100.0

day, with two level control serums called Precicontrol ClinChem Multi 1 (PCCM 1) and Precicontrol ClinChem Multi 2 (PCCM 2) which are produced by the same manufacturer during this period.

External quality control

Laboratory regularly participates in the Chemistry (C) external quality control programs provided by College of American Pathologists (CAP) for biochemical parameters during the period of study. Laboratory was accredited by Turkish Accreditation Agency (TURKAK) in 2004 and is undergoing regular annual inspection.

Table 5: The correlation analysis for all data and sub-groups.

	Correlation between tests			
	All data	C.1	C.2	C.3
AST-ALT	0.789*	0.651*	0.640*	0.596*
AST-GGT	0.461*	0.029	0.064	0.196*
ALT-GGT	0.570*	0.098	0.076	0.387*

Internal quality control

Coefficient of Variation (CV,%) values of operating parameters, inter-day values of the two level internal control samples obtained during the study period are given in Table 2.

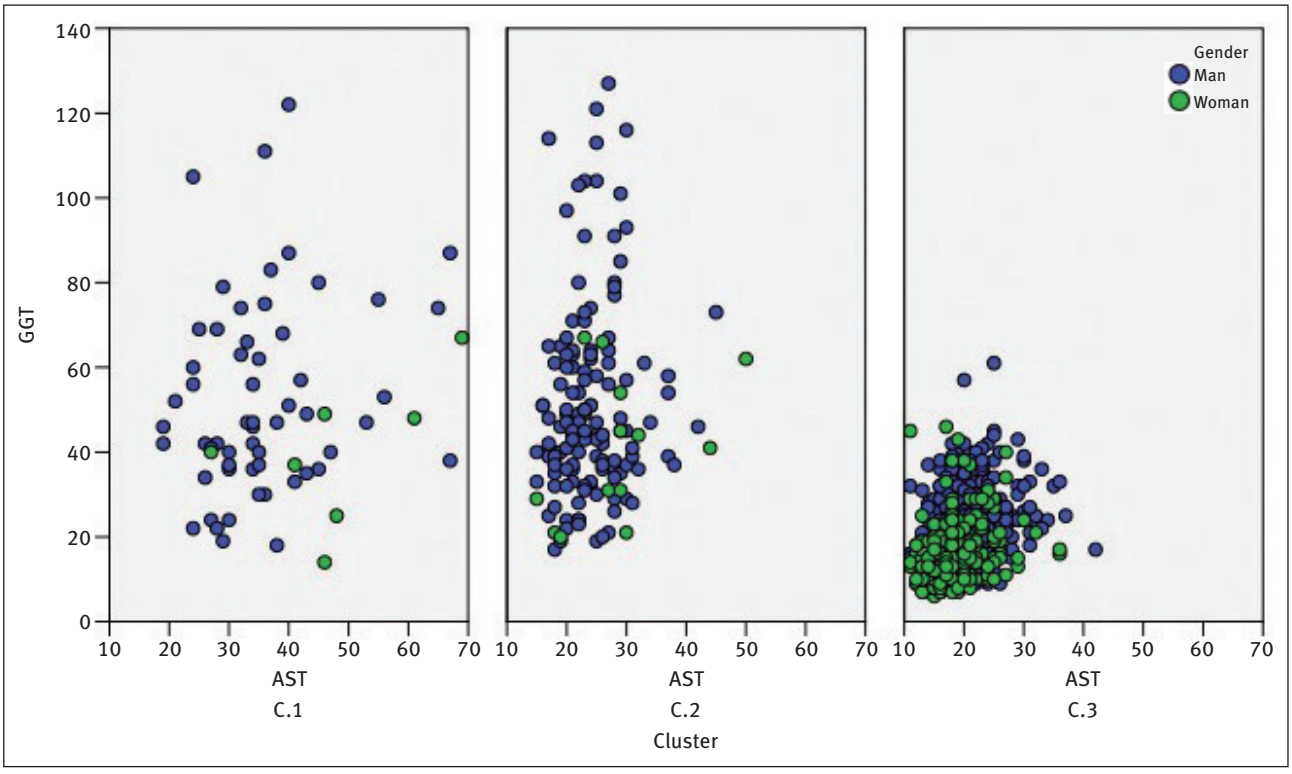

Figure 3: Scatter plot of GGT-AST for each cluster with gender.

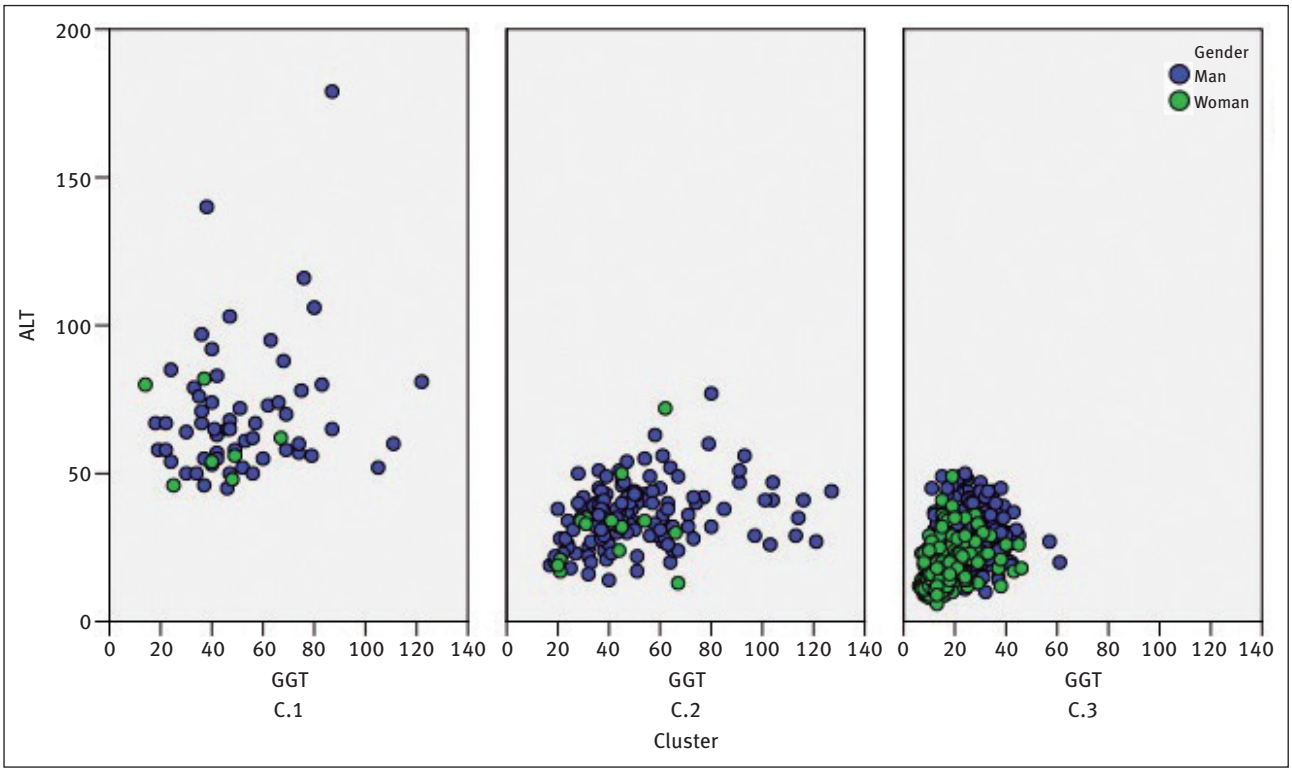

Figure 4: Scatter plot of GGT-ALT for each cluster with gender.

Data analysis and statistical methods

All of the analysis were performed with R.3.1.0 software. Descriptive statistics (mean, median, standard deviation, minimum and maximum values) are given for all of the parameters. Whether the data indicate normal distribution was tested with the Kolmogorov-Smirnov Test. Comparison of ALT, GGT and AST levels of each cluster analyzing has been performed by Kruskal Wallis test. Multiple comparisons have been done by Dunn's tests. Correlation between numerical parameters have been evaluated by Spearman Correlation Coefficient. The instructions in the NCCLS C28-P3 guide have been complied with in the analysis.

The most significant assumption in determining the reference limit: is of values necessity in the data set to be a homogenous group. This means that each sample comes from the same probability distribution. Some data sets contain the observations that do not fit into this interval. These values are called "outliers". Outlier data exclusion: if the data is skewed, outliers are excluded with Tukey method after logarithm transformation [6]; since the measured parameters did not have a normal distribution according to Kolmogorov-Smirnov test, the reference interval was computed using nonparametric method after excluding the outliers. The lower and upper reference intervals were defined as 2.5th and 97.5th percentiles of the distribution of values respectively, and 95% confidence limits were computed by nonparametric method for each reference limit as well. All of the analysis have been performed at the 5% level of significance.

Results

The descriptive statistics of a total of 883 individuals, 610 of which are male, 273 are female, who constitute the reference group, and the subgroups derived from this group by clustering analysis are given in Table 3. The whole data is divided into 3 clusters after the cluster analysis. The distribution of individuals according to the gender in these clusters are given in Table 4. When ALT, AST and GGT levels of three clusters statistically compared after Kruskal Wallis test and subsequent multiple comparison test (Dunn's test), all of the groups have been observed to be different from each other ($p=0.001<0.05$), Chi square=C1: 130.936, C2: 145.278 C3: 174.575). Correlations between tests for each sub-group are given in Table 5. Accordingly, when the correlations obtained with the whole data are considered, between AST-ALT a correlation of 78.9%, between AST-GGT a correlation of 46.1%, between ALT-GGT a correlation of 57% is observed. ($p=0.001<0.05$) However,

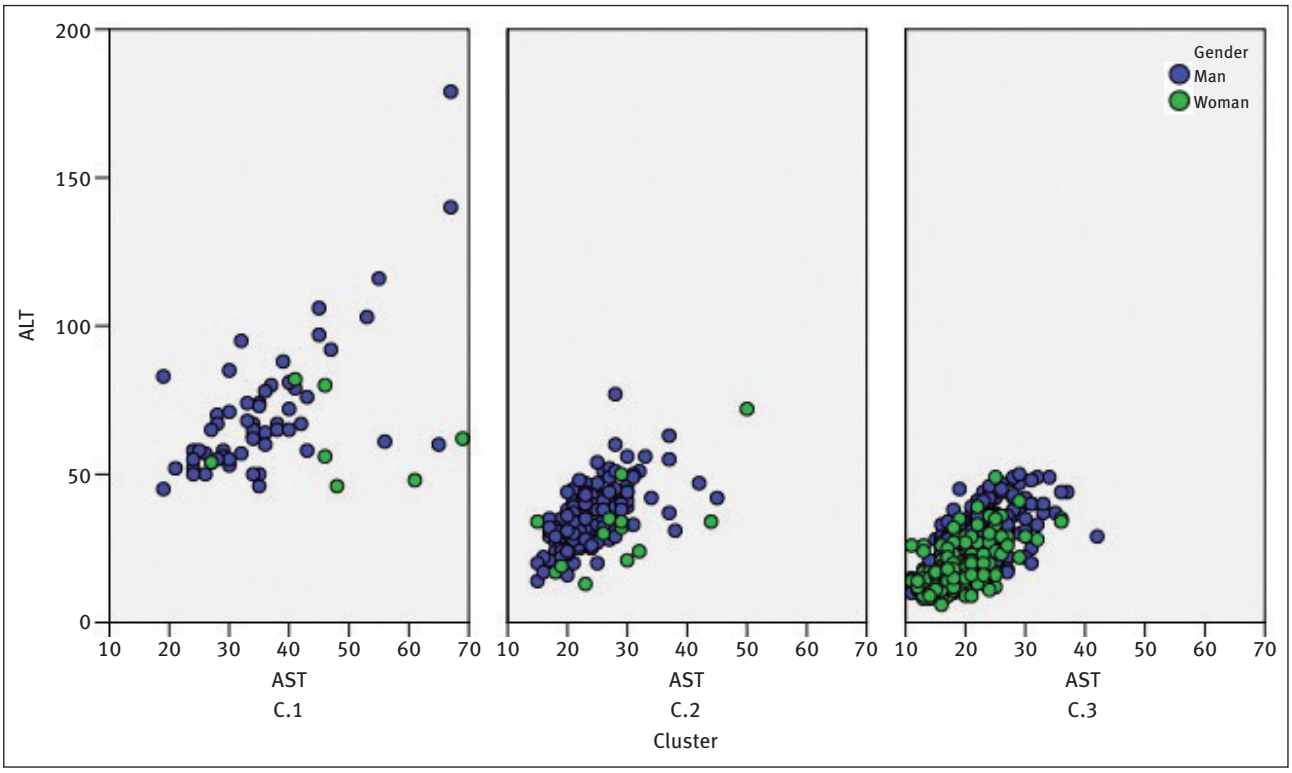

Figure 5: Scatter plot of ALT-AST for each cluster with gender.

when the sub-groups are observed these correlations are not statistically significant, except ALT-AST correlation.

Discussion

The most interesting aspect of the results obtained in this study is that the C3 might be "the real reference population". Because the laboratories can calculate the local RIs from their database, as proposed in the IFCC guide. A check-up individual known to have no complaint, with negative USG result is normal to be considered healthy. However, individuals who might be ignored and might not be called the statistical outliers, will able to be determined based on the correlation between the tests owing to the approach specified in our study.

When similar studies in the literature are examined, the most important point missed is analyzing the tests one by one in the outliers or RI calculation [17,20]. In the related studies, we think that one of the reasons of the difference especially in the region-based comparisons could be not analyzing the correlations between tests. When the RIs obtained within our study are examined, it can be observed that they have narrower intervals compared with the ones in the other studies. However, we have also observed wider RIs in the subgroups. This will be helpful in determining our "real reference population". The other important remarkable point in the subgroup based RIs calculation, which is the main purpose of the study, is that the RIs of the "real reference population" are different from the intervals in the subgroups. The most important reason of this is that the current recommended methods make a general calculation of RIs by ignoring the subgroups. The outliers analysis conducted on the basis of single tests within the whole data gives different results when recalculated by considering the correlations between tests. In this study, the correlation between ALT and AST with GGT decreases to lower levels in C1 and C2. Gender based scatter plot results obtained for each set are shown in Figure 3, 4 and 5. Accordingly, it was observed that the distribution of individuals in the C3 are more homogeneous, respectively. RIs of two approaches are given in Table 6. According to these results C1's reference intervals observed narrower when compared with C2 and C3. RIs obtained for C2 and C3 groups were observed especially to be larger and tend to be increasing.

By their nature, clinical sciences should not diagnose based on a single variable. Therefore, as in other studies in the literature, tests should be evaluated by considering together, not separately [1–8,16,17,20]. In our study, RIs of the individuals aged 18–70 were identified by a new

Table 6: Reference intervals obtained after classical method and cluster analysis.

Limits with. (U/L). (%95 CI)	MALE (n=610)		FEMALE (n=273)		M+F (n=883)	
	LL	UL	LL	UL	LL	UL
RI's at Düzen Laboratory-AST	10	40	10	32	0	34
AST-CAP	14.1 (13.3–15.1)	38.0 (36.0–41.1)	12.2 (11.2–13.1)	29.0 (27.3–30.1)	13.0 (12.0–13.1)	36.0 (34.3–37.2)
C.1	18.8 (17.9–19.1)	44.1 (41.2–46.2)	13.2 (12.9–14.0)	32.2 (31.5–33.4)	17.1 (17.0–17.5)	38.1 (38.0–41.2)
C.2	19.1 (19.0–21.0)	45.1 (45.0–46.1)	13.9 (13.0–13.7)	33.5 (33.2–35.7)	18.1 (18.0–19.3)	42.4(42.0–45.6)
C.3	13.1 (12.8–13.3)	31.4 (31.1–35.2)	15.0 (11.1–13.1)	26.1 (26.0–28.1)	13.9 (13.2–14.1)	28.6 (27.8–31.2)
RI's at Düzen Laboratory -ALT	8	41	6	33	10	49
ALT-CAP	12.9 (12.1–13.2)	64.0 (60.4–67.1)	9.1 (8.0–9.2)	35.2 (34.8–36.2)	10.0 (9.8–11.0)	56.1 (54.2–58.4)
C.1	16.7 (15.3–17.3)	65.1 (65.0–66.4)	12.4 (11.3–15.1)	38.5 (37.9–39.5)	14.3 (13.2–15.1)	59.3 (58.1–60.4)
C.2	17.2 (17.0–18.3)	67.1 (66.7–69.8)	13.5 (12.7–14.5)	40.1 (39.0–42.4)	15.1 (14.6–16.1)	60.3 (58.1–61.4)
C.3	14.1 (13.9–14.8)	61.0 (60.4–63.2)	10.1 (10.0–10.7)	33.6 (33.1–34.2)	12 (11.8–13.0)	52.0 (51.2–53.5)
RI's at Düzen Laboratory-GGT	8	61	5	36	0	70
GGT-CAP	12.1 (11.3–13.1)	76.95 (71–83)	7.0 (6.91–8.0)	33.5 (29.2–38.3)	9.0 (8.0–9.1)	68.2 (64.6–74.3)
C.1	14.1 (14.0–15.1)	79.1 (79.0–82.3)	8.1 (8.0–9.1)	35.2 (34.7–37.6)	10.3 (10.0–10.5)	69.4 (67.2–75.2)
C.2	15.1 (13.9–16.1)	80.2 (80.0–80.6)	8.9 (8.7–9.4)	36.1 (35.5–37.1)	10.9 (10.5–11.0)	71.5 (71.0–75.7)
C.3	12.5 (11.9–13.1)	71.2 (70.4–74.6)	8.2 (7.7–8.3)	31.2 (31.0–32.3)	13.2 (12.0–13.6)	54.4(52.3–59.2)

CAP: Classical approach; C: Cluster

approach. However, the same analysis are planned to be repeated on every age or age-intervals with more individuals in future studies.

Consequently, we think that check-up individuals can be considered as reference individuals in the RIs studies, but researchers should not use the whole data that they have. They have to perform clustering analysis. In this way, the differences especially in the countries such as Turkey, which have regions with different environmental factors, will be revealed. And it is obvious that the commercial firms need to design experiments by taking these differences into consideration when proposing RIs for their kits.

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

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