The body skeleton contains about 99% of the total calcium, small amounts of this one are found in plasma and extravascular fluid. Very low calcium intake may contribute to the poor development of infants and rickets in children [1].

Demineralization processes in the oral cavity are strongly influenced by the salivary factor. The protective role of saliva is due to the salivary calcium concentration [2]. A serum total calcium test measures the total calcium in blood [3]. For the accurate interpretation of the results, knowledge of normal reference ranges is crucial as there may be differences from one area to another, depending on ethnicity, gender, age, mother diet in pregnancy, quality of the children’s diet, climate and seasons.

Establishing reference intervals represents a powerful tool in laboratory medicine, providing valuable information for the correct interpretation of laboratory results and helping clinicians to make correct decisions [4, 5]. Determining the reference intervals is difficult to perform, being time consuming and expensive [4].

Hoffmann indirect method for determining the reference range can be used by any medical analysis laboratory which has an electronic stored database. This method is very convenient taking into consideration the fact that it eliminates the need to recruit healthy individuals as it uses the data that has already been collected and is available in the database [6].

Determining the reference range using other methods require the recruitment of healthy individuals from different age groups. Recruiting children and adolescents is particularly difficult because of all the changes that occur during their growth and development [7, 8].

Also collecting samples is difficult, especially from little children and infants, as they need to be feed at short time intervals. In addition, healthy volunteers need to be motivated to participate to such test, requiring their informed consent. The organizational costs would be very high [9].

**Experimental part**

This study was conducted in the Biophysics and Biochemistry section of the Faculty of Medicine in Sibiu, in collaboration with the S.C. Vladutiu & Garabedian S.R.L. Clinic in Medias. Our team aimed to determine its own reference intervals for children and adolescents in Medias area (Romania) based on the laboratory equipment, working reagents and protocols used by us. We started from the hypothesis that there might be differences from other parts of the country or EU depending on the relief and climate which can influence the quality of food, depending on the education and traditions which can also influence some eating habits or depending on the diets of pregnant women and lactating mothers.

The reference intervals obtained by us were compared with the ones in CALIPER studies [11, 12].

The sample of patients performed at the S.C. Vladutiu & Garabedian S.R.L. Clinic of Medias, included 3217 results from blood samples taken five years from children and adolescents.

The study was made possible by the use of results of the electronic database of the laboratory [7, 10].

All samples of blood to determine serum calcium were collected by vein puncture into vacutainers without anticoagulant. The blood collected was allowed to stand at room temperature then centrifuged at 3000 rpm for 10 min. The analysis of serum calcium levels was performed on Konelab analyser. This uses Arszenoz I reagent [11], which together with calcium ions form a strongly coloured complex which is measured by spectrophotometry at 660 nm wavelength.

Konelab analyser is calibrated and tested with calibration substance and control serum before performing the actual tests. The examinations are conducted only if calibration is within normal parameters [4]. As suggested by the manufacturer, daily, weekly and monthly maintenance, of the analyser is made.
Hoffmann indirect method uses the results from the database, subjects having minimal pathology, taking into account 5% up to 95% of the values obtained, the values being selected randomly [6]. Two hypotheses are considered [6]: the first involves that in the database of a clinical analysis laboratory, data for a given analyse forms a Gaussian distribution and the second says that most of the laboratory results represent normal individuals (healthy).

The results used for the study were obtained from the electronic archive of the laboratory.

There were analysed data from 3217 patients aged 1 month and patients over 18 years old, divided into four categories, namely: 1-5 years of age; 6-10 years, 11-14 years, and 15-20 years. Age groups were classified according to the organization of the Clinic, taking into account the age groups of the children. First, aberrant values were eliminated, according to Hoffmann method by using the Chauvenet criterion [6, 10].

After eliminating these values, cumulative frequency was established.

Cumulative frequency is the arithmetic sum of frequency of a result of analysis FXj, ordered by Xj [6, 10]. FXj is equal to the ratio between the number of occurrences of a result in the data set and the total number of data, all multiplied by 100%.

Cumulative frequency depending on calcium concentration values was represented on the chart and there was established the portion of the line linearity and maximum of deviation.

The best equation of the regression is:

$$Y_i = X_i + A \cdot B + E_i$$

where: A is the slope, B is the intercept of the line and Ei is the error [6].

By solving the regression equation, there have been determined the maximum ($V_{max}$) and minimum ($V_{min}$) values, given that the values must be within the range 2.5% and 97.5% [10].

$$V_{max} = A \cdot 97.5 + B$$ and $$V_{min} = A \cdot 2.5 + B$$

Reference intervals obtained were then compared with those of CALIPER (Canadian Laboratory Initiative in Pediatric Reference Intervals) [11, 12] because the studies CALIPER used Arsenazo III reagent, as in our studies.

Results and discussions

Using these data sets, we established reference intervals for serum calcium in the selected patients per age groups. Figure 2 indicates total calcium reference ranges for the age group of 1-5 years old. In the third figure, there are emphasized total calcium reference ranges for the age group of 6-10 years old. The fourth figure contains the graph showing total calcium reference ranges for the age group of 11-14 years old, and the figure 5 reflects the graph showing total calcium reference ranges for children and adolescents of 15-20 years old.

Regression functions are marked on the charts and the reference ranges for serum total calcium was calculated using the indirect Hoffmann method ($V_{min}$ and $V_{max}$) [5]. There was made a comparison between the Calculated Reference Ranges and References Reported from CALIPER.

There was not possible to calculate reference intervals for children less than 1 year old because sufficient data was not available, minimum 120 data being needed according to Hoffmann method.

For the age group between 1-5 years old, following the calculations, there were obtained reference ranges between 2.15 - 2.60 mmol/L for serum calcium levels, while in the studied literature, the reference intervals for this age group are relative to 2.30 to 2.60 mmol/L; so we can say that the lower limit were found to be slightly lower in the children within the current study.

Regarding the age group between 6-10 years old, there have been obtained reference intervals of 2.18 - 2.58 mmol/L, while in the CALIPER results, the reference intervals for this age group are relative to 2.30 to 2.60 mmol/L.

For adolescents between the ages of 11-14 years, with linear regression and regression function shown in figure 4, we obtained reference intervals ranging from 2.18-2.60 mmol/L Comparing the results of our ranges of reference established by CALIPER (2.20 - 2.60 mmol/L), cannot see significant differences.

For adolescents between the ages of 11-14 years, with linear regression and regression function shown in figure 5, we obtained reference intervals ranging from 2.12 to 2.52 mmol/L. Comparing our results with the reference interval stated in CALIPER (2.20 - 2.60 mmol/L), it can be seen that the lower and upper limit were found to be slightly lower in the adolescents within the current study.

It should be noted that we have not calculated whether the difference between the values obtained by us and those found in the literature is statistically relevant because studies were not conducted in the same manner, using the same laboratory equipment.
For all age group we obtained values almost identical with those stated in CALIPER reports [11, 12]. Reference intervals are specific for the population of Medias area, and for the method used in our laboratory. Although these are similar to those reported from CALIPER, additional focus is needed to establish the reference ranges personalized for our laboratory, conducting a study on a controlled population.

**Conclusions**

The values obtained in the current study need to be validated and only then can be used in our daily activity. The validation process needs healthy volunteers and because they are infants, informed consent is also necessary. These limitations must be addressed in order to use the results and move forward with this study.

The most critical steps in determining the reference values are those related to the selection of healthy people, therefore Hoffmann indirect method is used, which serves to establish reference values using data from the database.

The obvious advantage of this approach is that it eliminates the need to recruit healthy people instead taking advantage of hospital data which has already been collected and is available.

The method presented by us for determining the reference intervals has the advantage of using existing data from our laboratory’s electronic database and there is no need to recruit new healthy individuals.

All studies presented in specialized literature have been conducted in other countries, using different apparatus, reagents and methods, having different health policies and patients having different life-styles. All this factors could greatly affect the results. Despite those differences we mention that the results obtained are similar or identical with the ones presented by CALIPER studies.

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