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ORIGINAL ARTICLE

Reference intervals for growth arrest-specific 6 protein in adults

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ABSTRACT

The objective of this study was to establish reference intervals for growth arrest-specific 6 (GAS6), a vitamin K-dependent protein, in human adult plasma according to the Guideline of Clinical and Laboratory Standards Institute (CLSI) C28-A3. Blood samples were collected from 308 healthy volunteers aged 18–72 (157 female, 151 male). A non-parametric approach was used to calculate the reference interval. The plasma GAS6 reference interval was determined, with 90% confidence interval: the lower limit (2.5 percentile) was 2.5 (1.9–3.1) μg/L and the upper limit (97.5 percentile) = 18.8 (18.0–22.3) μg/L. Harris-Boyd's test did not suggest partitioning by age or gender: medians for males [7.8 (5.8–10.7) μg/L] and females [9.9 (7.1–13.5) μg/L]. Three age-subgroups were tested: 18–29 years ($n = 168$); 30–44 years ($n = 73$); 45–72 years ($n = 67$). The intra- and inter-assay variations were 12.6% (mean, 5.2 ± 0.7 μg/L) and 14.0% (mean, 9.2 ± 1.3 μg/L), respectively. The mean recovery was 104%. This study reports plasma GAS6 reference intervals established first according to the guideline of CLSI C28-A3.

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Introduction

Growth arrest-specific 6 protein, GAS6, was originally identified in fibroblasts as a gene whose expression was up-regulated under the conditions of serum starvation [1]. GAS6 is a vitamin K-dependent protein that shows 43% amino-acid sequence identity with protein S, a negative regulator of blood coagulation. However, GAS6 lacks its anticoagulant activity [2]. Unlike vitamin K-dependent coagulation factors [3,4], the main site of synthesis for GAS6 is not the liver. A number of *in vitro* studies have demonstrated that GAS6 is expressed in several kinds of human cells and tissues, such as normal and malignant hematopoietic cells [5], endothelial [6] and vascular smooth muscle cells [7], Sertoli cells [8], and brain tissue [9]. GAS6 undergoes gamma-carboxylation to become biologically active in the presence of vitamin K. *In vitro* studies have shown that peripheral carboxylation is inhibited by administration of warfarin [10].

GAS6 has been implicated in numerous physiological processes, including cell migration [11], adhesion [12], and cell growth and survival [13]. It is the ligand for Tyro3, Axl and Mer (TAM receptors), which are members of the tyrosine kinase family [14].

Based on the results of cell culture studies and animal models, there is some evidence that GAS6 may be implicated in inflammation [15], autoimmune [16], vascular and kidney diseases [17]. It has been shown that expression of GAS6 and its receptors in tumor tissues is implicated in malignancies, such as lung [18], gastric [19], breast [20] and colon [21] cancer. It is evident that GAS6 is linked to some physiological and physiopathological conditions, but its precise physiological role has yet to be clarified. In order to interpret the values of plasma GAS6 concentrations in diseases, a reliable reference interval needs to be established.

Some research groups from different countries have developed novel ELISA methods for the assessment of plasma GAS6 concentrations [22–24]. Two have reported plasma GAS6 concentrations for healthy sample populations [22,23]. However, sample sizes for these studies were 94 participants (57 female, 37 male) [22], and 61 participants (41 female, 20 male), respectively [23], less than the minimum 120 subjects (120 female and 120 male) recommended by CLSI (Clinical and Laboratory Standards Institute) C28-A3 for reference interval studies [25]. Clauser et al. assessed a healthy group consisting of 88 females and 94 males [24]. Though the closest to date, this number still fell well below the recommended minimum.

Previous clinical studies [26–28], including ours [29–31], that have included control groups as part of their investigations, have observed GAS6 concentrations that are highly variable. Since the main objective of these studies was not to estimate the reference interval of plasma GAS6, the selection criteria for control group participants deviated significantly from the selection criteria typically employed in the present study. We needed to establish selection criteria across a

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number of variables including age, vitamin K status, smoking, alcohol and drug intake, infection, and chronic diseases.

In order to allow for accurate interpretation of plasma GAS6 concentrations, reference intervals developed using systematic and standardized processes such as those outlined by the Guideline of CLSI and IFCC (International Federation of Clinical Chemistry) are necessary [25]. Because these intervals may vary with age, ethnic origin, socio-demographic characteristics, and environmental context, they are population-specific [32–34]. The main objective of this study was to determine for the first time the reference interval of plasma GAS6 in adults according to the recommendations of IFCC and CLSI C28-A3.

Materials and methods

Study population and sample collection

A total of 308 healthy adult volunteers participated in this study (aged 18–72 years; 157 females and 151 males) by donating a plasma specimen for GAS6 measurement. The study participants gave written informed consent. The study protocol followed the ethical guidelines of the Helsinki Declaration of 1975, as revised in 1983 [35], and was approved by the local ethics committee. All volunteers enrolled in the study filled out a health questionnaire, which was prepared in accordance with the protocol in the Guideline of CLSI C28-A3 [25]. As a part of the Mediterranean lifestyle, their habitual food consumption included fruit and green leafy vegetables, a good source of vitamin K. Volunteers were excluded if they had systemic disease or infection. None of the study participants were on anticoagulation therapy or vitamin K supplementation. Other criteria for exclusion were: infection, smoking, regular use of alcohol, drugs or vitamins, chronic disease or abnormal blood pressure. Those who had undergone surgery or drug intake in the previous week were also excluded.

Venous blood samples were collected into vacuum tubes containing 3.2% Na-citrate between 08:00 and 10:30 h following an overnight fast. The plasma aliquots were stored at −80 °C after centrifugation at 2300 *g* for 15 min at room temperature. Icteric, hemolyzed or turbid samples were not included in the study.

ELISA for plasma GAS6

The human GAS6 sandwich ELISA development kit (R&D Systems Inc., Minneapolis, MN, USA) was used which was designed for the analysis of cell culture supernatants. When we started this study, it was not optimized by the manufacturers for analysis of human plasma. The method has been optimized, validated and used for our previous publications [29–31]. A Substrate Reagent Pack (Color reagent A&B) was used from R&D Systems.

Briefly, microtiter plates (Nunc, Maxisorp U, 96 wells) were coated with 100 µL capture antibody (mouse anti-human Gas6, R&D Systems) and incubated overnight at 4 °C. The next day, the wells were washed five times with a washing buffer of PBST (phosphate buffered saline tween-20, 0.05% Tween-20 in PBS), pH 7.4, and then blocked with 5% BSA (Bovine serum albumin) (Fraction V, Roche) in PBST for 2 h at room temperature. After five washes with the washing buffer, 100 µL of diluted calibrators or samples (1/40 in PBST containing 1 mM EDTA and 1% BSA) were added to the wells and incubated for 1 h at 37 °C. The biotinylated secondary antibody (4 µg/mL, goat anti-human GAS6, R&D Systems) was added to the wells, followed by washing steps for 1 h at 37 °C; 100 µL of streptavidin peroxidase conjugate (400 µg/L in PBST) was added to each well and incubated for 20 min at room temperature. The wells were washed five times with a washing buffer, and a 100 µL equal mixture (v/v) of color reagents A and B was added to each well. Wells were incubated for 15 min at room temperature. The reaction was then stopped by the addition of 50 µL of stop solution (1 mol/L H_2SO_4). The absorbance was measured at 450 nm on a microplate reader.

Preparation of GAS6-depleted plasma

A matrix similar to plasma, but depleted of GAS6 was prepared. This GAS6-depleted plasma was created by adsorption of vitamin K-dependent plasma proteins with insoluble barium salts and then removal of GAS6 by immunoprecipitation using anti-GAS6 antibodies.

The adsorption characteristic onto insoluble barium salts is unique to the vitamin K-dependent proteins. Barium citrate was precipitated by the slow addition of one-tenth volume of 1.0 mol/L $BaCl_2$ to plasma [3,4]. The suspension was stirred gently for 60 min and then centrifuged at 2300 *g* for 10 min. The supernatant was subjected to immunoprecipitation with anti-GAS6 antibodies. Dot-Blot was used to check the effectiveness of depletion.

Intra-assay variability

Intra-assay variability was tested by measuring plasma pools of samples 10 times in a run. Mean, standard deviation, (SD) and coefficient of variation (CV) values of the measurements were calculated.

Inter-assay variability

Plasma pools were prepared from samples containing 3.2% Na-citrate drawn from donors; aliquots were stored at −80 °C. GAS6 concentrations within these pools were measured over 10 days, together with other samples. The mean, SD and CV values for different days were determined.

Recovery

Recovery analyses were performed in order to examine the bias of the ELISA method. It was evaluated by adding GAS6 (40, 20, 10, 5 or 2.5 µg/L) to human plasma.

GAS6 calibration curve

Recombinant human GAS6 (R&D Systems, Minneapolis, MN, USA) was dissolved into GAS6 depleted plasma and

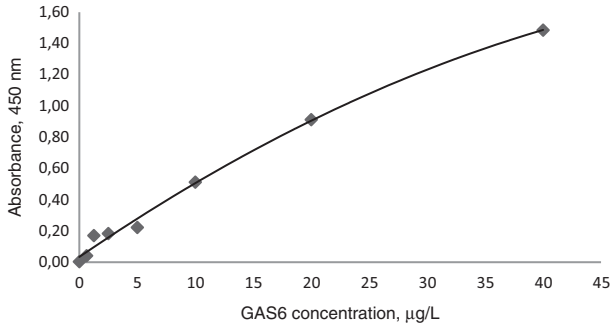

Figure 1. GAS6 calibration curve.

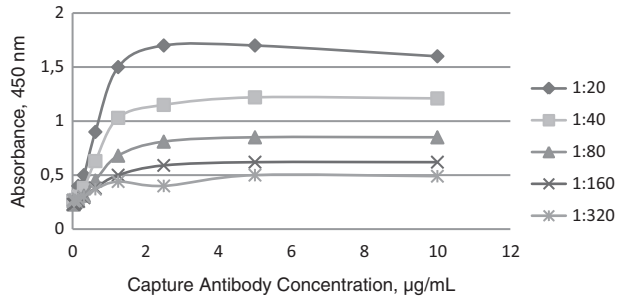

Figure 2. Optimization of ELISA: Comparison of dilution ratios of samples against capture antibody concentrations.

used as the main calibrator. GAS6 calibration curve was prepared using the serial dilutions of the calibrator parallel to the dilutions of a high patient sample.

Statistical analysis

Data were analyzed according to the CLSI C28-A3 guideline [25]. Dixon's Test was used for examination of outliers [36]. The Kolmogorov-Smirnov test was used to evaluate the closeness of the data to a normal distribution. Age and gender partitioning decisions were made according to Harris and Boyd's test [37]. Data were combined and reevaluated when the results of Harris and Boyd's test did not indicate partitioning. The nonparametric rank method was used to calculate the reference interval for each group with a sample size $\geq$120. The Mann-Whitney U test was used for comparing continuous variables between two groups. The Kruskal Wallis test was used for comparing continuous variables between three groups. A p value of <0.05 was considered to be significant. Bonferroni correction was used for post-hoc comparisons.

Results

In order to optimize the ELISA method, the following parameters were tested: capture antibody concentration; dilution solution; dilution ratio of samples and calibrators; blocking agent [BSA or non-fat dry milk (NFDM)], and incubation time and temperature. When 1 mmol/L ethylenediaminetetraacetic acid, EDTA, and 1% BSA in PBST were used as the dilution solution for calibrators and samples, we obtained a meaningful calibration curve (Figure 1). Without EDTA, all absorbance values for samples were very low.

Figure 2 shows the comparison of different dilution ratios of samples against different concentrations of the capture antibody. The slope was at a maximum at the dilution ratio of 1:20. Since the slopes of the ratios of 1/40 and 1/20 were very close to each other, the optimal dilution ratio for the samples and calibrators was selected as 1/40.

Blocking of unoccupied microtiter plate binding sites was achieved by incubating the plates with 5% BSA in PBST for 2 h at room temperature. When NFDM was used instead of BSA, a decrease in the slope of the calibration curve was observed. For this reason, 5% BSA in PBST was chosen as the blotting solution.

In summary, the following optimal conditions were identified: Dilution solution for samples and calibrators: PBST containing 1 mmol/L EDTA and 1% BSA Dilution ratio of samples and calibrators: 1/40 Capture antibody concentration: 4 µg/mL Blocking solution: 5% BSA in PBST Incubation time during antigen antibody interaction (both capture and detection antibodies) was 1 h at 37 °C.

Intra-assay variation was 12.6% at a mean concentration of 5.2 ± 0.7 µg/L. When GAS6 concentration of a pool was measured at each run together with other samples, inter-assay variation was found to be 14.0% at a mean concentration of 9.2 ± 1.3 µg/L. At a higher concentration (27.2 ± 3.2 µg/L) inter- and intra-assay CVs were 15.8% and 13.4%, respectively. The mean recovery was 104% for the method. The limit of detection was 1.2 µg/L.

GAS6 concentrations were measured in a reference group consisting of 308 individuals (18–72 years old). The histogram of the results of female ($n = 157$) and male ($n = 151$) are shown in Figure 3. The Kolmogorov-Smirnov test indicated that the data were distributed normally for females but not for males or the whole population.

When the extreme values of the whole population, female and male, group by group, were examined separately, no extreme values were observed for the whole group or females; however, one extreme value was observed for males. Since the ratio D/R for the most extreme GAS6 concentration was less than 3, the extreme value was not accepted as an outlier, it was included in the group.

The Harris and Boyd's test did not indicate partitioning between males and females. Since $z < z^*$ (3.06 < 3.4), the groups were not separated but combined ($n = 308$) and reevaluated. A non-parametric approach was used to calculate the reference intervals. The following limits of the central 95% reference interval, along with the 90% confidence interval (in brackets), were obtained:

Lower reference limit (2.5 percentile) = 2.5 (1.9–3.1) µg/L

Upper reference limit (97.5 percentile) = 18.8 (18.0–22.3) µg/L

In contrast to the Harris and Boyd's test, the Mann-Whitney U test indicated a difference between males [7.8 (5.8–10.7) µg/L] and females [9.9 (7.1–13.5) µg/L; median (25–75% percentile)] ($p < 0.05$).

Three age groups were compared: 18–29 years [8.7 µg/L (5.8–13.1); median (25–75% percentile), $n = 168$] and 30–44 years [8.2 ± 3.7 µg/L; mean ± SD, $n = 73$] and 45–72 years [10.4 ± 3.7 µg/L; mean ± SD, $n = 67$] (Figure 4). A Kruskal-

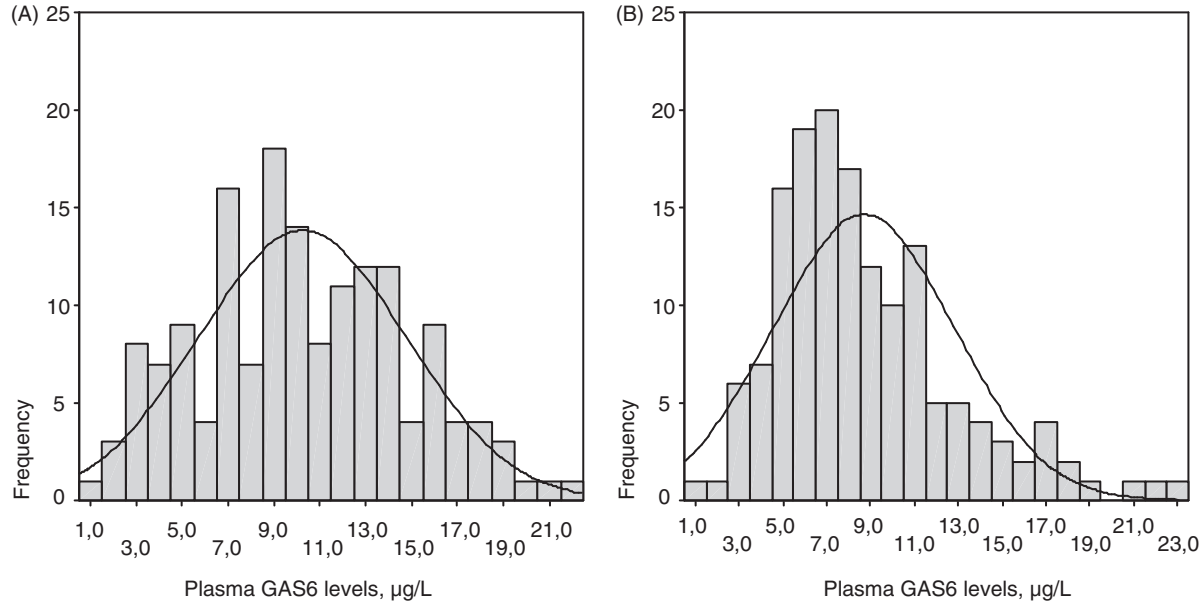

Figure 3. Distribution of plasma GAS6 concentrations in reference samples. (A) Females ($n = 157$); (B) Males ($n = 151$).

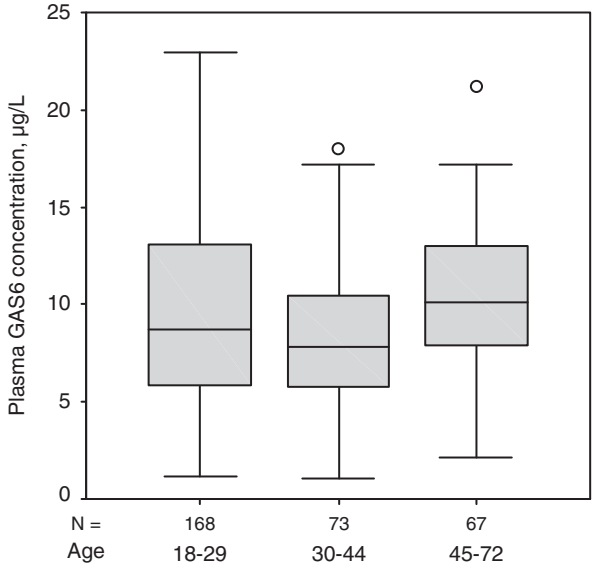

Figure 4. Box-and-Whisker plots of plasma GAS6 concentrations of the age groups.

Wallis test indicated a significant difference between the three age groups ($p = 0.004$). The Mann-Whitney U test with the Bonferroni correction indicated a difference only between the 30–44 age group and 45–72 age group ($p < 0.0167$) but not between the other age-subgroups. To compare two subgroups the CLSI guideline recommends use of Harris-Boyd's test. In this study, it did not indicate partitioning by age. Following the recommendations of CLSI guideline and depending on results of Harris Boyd's Test, we combined the data from three age groups.

Discussion

Studies evaluating plasma GAS6 concentrations have offered variable results, lacking a general consensus. Using a home-made capture antibody, Balogh et al. (2005) developed an ELISA method and reported a reference interval of 13–23 µg/L (0.16–0.28 nmol/L) ($n = 94$), which was not affected by participant gender or age (22–65 years) [22].

Alciato et al. (2008) developed an ELISA test with commercial antibodies and found a mean plasma GAS6 concentration of 20.3 ± 3.8 ng/mL ($n = 61$) [23]. The authors observed no significant differences between genders, and across the two age groups tested: 20–35 years and 54–79 years. In the present study ($n = 308$) age range was 18–72, the majority of participants were between the ages of 18 and 29. The Harris and Boyd's test did not indicate partitioning between males and females, similar to the other studies [22,23]. The variations between these studies may be explained by the difference in population size, as well as differences in analytical techniques.

Some clinical studies reported plasma GAS6 concentrations of their control groups. Ekman et al. (2010) used the methods developed by Balogh et al. to investigate the relationship between GAS6 and abdominal aortic aneurysm [27]. In the control group ($n = 141$), the concentration of plasma GAS6 was measured as 11.9 (4.6–23.8) µg/L. In a subsequent study by the same group, GAS6 concentrations of 0.20 nmol/L (0.1–12.4) were recorded for the control group ($n = 204$) [38]. The age range of participants (67–78 years) was higher than the previous one, 107 of whom were hypertensive and an additional 24 were smokers. In a study of Hung et al. mean plasma GAS6 concentration of 14.3 ± 0.7 µg/L were reported for the control group (43 male/53 female, age 50.2 ± 1.54 years) [39]. In their study of sepsis, Borgel et al. (2006) reported a mean plasma GAS6 concentration of 54 µg/L in a control group of 30 people [26]. Because these studies have been conducted primarily for the purposes of evaluating GAS6 levels in disease populations, the selection criteria employed for control group participants were often less stringent. Methodological differences, age, gender, and locality of population are likely to contribute to the difference.

Clauser et al. observed that GAS6 concentration varied according to gender. The authors reported plasma GAS6 concentrations of 52 μg/L in males ($n = 94$) and 63.8 μg/L in females ($n = 88$) for healthy volunteers (18–38 years) [24]. They did not use the Harris Boyd's test for comparison of females and males. In the present study, only the Mann-Whitney U test indicated a difference by gender, but not the Harris Boyd's test, which did not suggest a clinically significant difference in terms of age. Only the Mann-Whitney U test with Bonferroni correction indicated a difference between the age-subgroups 30–44 years and 45–72 years ($p < 0.0167$). Differing results from Mann-Whitney U test and Harris and Boyd's test are not surprising because the Mann-Whitney U test is more sensitive in detecting changes, even such a small difference could be irrelevant in clinical practise. As discussed in the CLSI guideline 'when more than two subclasses are compared, the issues become more complicated' and 'further research appears to be needed in this area'.

The calibrators used in different studies bring about variation of the results. The lack of any reference material and methodology for GAS6 makes it difficult to compare the reported results for GAS6. Better traceability and standardization of GAS6 measurement is needed.

One limitation of this study regarding age ranges is that the number of participants is not equal in the three different age groups. The number of individuals between the ages of 18–29 years was 168 whereas the numbers of individuals between the ages of 30–44 ($n = 73$) and 45–72 ($n = 67$) were less than 120. With a larger population, more definite results could be reached. More research needs to be conducted to determine whether plasma GAS6 concentrations correlate with age.

In conclusion, this study reports the plasma GAS6 reference intervals for adults according to the guidelines set out by CLSI and IFCC. We observed a lower reference limit (2.5 percentile) of 2.5 (1.9–3.1) μg/L and an upper reference limit (97.5 percentile) of 18.8 (18.0–22.3) μg/L. This represents the most comprehensive evaluation to date, covering the largest number of participants.

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Disclosure statement

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