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Short-term biological variation of serum tryptase

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Abstract

Objectives: Serum tryptase is a biomarker of mast cell activation. Among others, it is used in the diagnosis of anaphylaxis where a significant increase during the acute phase supports the diagnosis. When evaluating changes in biomarker levels, it is of utmost importance to consider the biological variation of the marker. Therefore, the aim of this study was to evaluate the short-term biological variation of serum tryptase.

Methods: Blood samples were drawn at 9 AM three days in a row from apparently healthy subjects. On day two, additional blood samples were drawn every third hour for 12 h. The tryptase concentration was measured in serum using a fluoroenzyme immunoassay (ImmunoCAP™, Thermo Fisher Scientific). Linear mixed-effects models were used to calculate components of biological variation.

Results: In 32 subjects, the overall mean concentration of tryptase was 4.0 ng/mL (range, 1.3–8.0 ng/mL). The within-subject variation was 3.7 % (95 % confidence interval (CI) 3.0–4.4 %), the between-subject variation was 31.5 % (95 % CI 23.1–39.8 %), and the analytical variation was 3.4 % (95 % CI 2.9–4.1 %). The reference change value was 13.3 % for an increase in tryptase at a 95 % level of significance. No significant day-to-day variation was observed ($p=0.77$), while a minute decrease in the serum concentration was observed during the day ($p<0.0001$).

Conclusions: Serum tryptase is a tightly regulated biomarker with very low within-subject variation, no significant day-to-day variation, and only minor semidiurnal variation. In contrast, a considerable between-subject variation exists. This establishes serum tryptase as a well-suited biomarker for monitoring.

Introduction

Tryptase is a trypsin-like serin protease produced mainly by mast cells [1]. During normal homeostasis the serum tryptase level is low, but tryptase is released upon activation of the mast cells and the serum level increases substantially [2]. The function of tryptase is not completely elucidated, but it is believed to promote numerous proinflammatory functions [2]. Elevated serum tryptase concentrations have been observed in several pathological conditions including systemic allergic reactions, and serum tryptase has proven valuable as a biomarker in anaphylactic reactions [3] as well as in non-anaphylactic conditions [4]. The tryptase level is a minor diagnostic criterion for mastocytosis, and it is widely accepted that an increased serum baseline tryptase concentration above 20 ng/mL is suggestive of mastocytosis [5]. Additionally, tryptase serves as a potential biomarker for some hematological malignancies [6].

In the diagnostic of anaphylaxis, serum tryptase is used as a biomarker in a retrospective manner to confirm the diagnosis [3]. During an anaphylactic reaction, the serum tryptase level increases throughout the acute phase of the reaction but rapidly returns to baseline after approximately 12 h [2]. Hence, it is important to know the acute serum tryptase level as well as the baseline level to avoid misinterpreting an important increase in acute tryptase in a patient with a very low concentration of baseline serum tryptase. An international consensus has proposed that a clinically significant acute increase in tryptase is defined as $120\% \times \text{baseline} + 2 \text{ ng/mL}$ [7]. This equation is widely used in the clinic, even though other equations have been proposed [3, 8, 9].

Although serum tryptase is a well-established biomarker, only sparse information is available on its biological variation (BV) [10, 11]. Knowledge of the BV is crucial when evaluating if an observed change in serum level is actually due to an anaphylactic reaction or caused by BV. Therefore, the aim of this study was to estimate the magnitude of the short-term BV of tryptase, and based on this, calculate the reference change value (RCV). This will help the interpretation of serum tryptase measurements in the clinic.

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Materials and methods

Subjects

For this study, apparently healthy subjects were included between June 2018 and October 2018 at Aarhus University Hospital, Denmark. Previously, in- and exclusion criteria together with a detailed presentation of the study cohort have been comprehensively reported [12]. In short, subjects were included if they were older than 18 years and did not have any signs or history of acute or chronic infections or inflammations. Subjects were excluded if they were pregnant, smoking, received any medical treatment, or had nightshift work or crossing of time-zones in the week before sampling.

The study was approved by the Central Denmark Region Committees on Biochemical Research Ethics [1-10-72-452-17] and conducted in accordance with the Helsinki Declaration. Written confirmed consent was obtained from all subjects before inclusion.

Study design

At 9 AM on three consecutive days, a blood sample was drawn. On the second day, blood samples were additionally drawn every 3 h during a 12 h period (at 12 PM, 3 PM, 6 PM, and 9 PM). Subjects were included in the study if the three 9 AM blood samples were drawn, even though they did not contribute with some or all the remaining blood samples. Throughout the study period, subjects were not allowed to drink alcohol or perform strenuous physical activity. Furthermore, they were not allowed to consume any food or perform low-intensity physical activity 1 h before every blood sampling.

Checklists for biological variation studies were considered when designing the study [13–15].

Blood sample collection and processing

Handling of blood samples have been reported previously [12, 16]. In brief, blood was collected in 10 mL serum tubes (BD vacutainer®) by venipuncture in the elbow. The serum tubes were set to incubate for 30 min before centrifugation at RT (10 min at 1,800 g). Afterwards, they were frozen at -80°C until analysis. All blood samples were obtained and handled by the same four skilled technicians.

Laboratory analysis

The quantification of serum tryptase was performed in November and December 2022 at Aarhus University Hospital, Aarhus, Denmark on a Phadia 250 Immunoassay Analyzer (Thermo Fisher Scientific, Massachusetts, USA) using the ImmunoCap™ sandwich immunoassay with fluorescence detection according to the manufacturer's instructions. Calibrators, reagents, and controls delivered from the manufacturer were used as recommended. Limit of quantification for the analysis was 1.0 ng/mL [17]. The within-laboratory precision during the analysis period was 7.8 % as measured by internal quality control. The laboratory participated in the external quality assessment schemes UK NEQUAS Tryptase and met the performance target reported by the assessment schemes in November 2022.

Samples were thawed at RT on the day of analysis without any prior thawing and were mixed well before loaded on the equipment. To minimize analytical variance, all samples from each individual were batch analyzed, and all analyses were performed by a single trained technician using the same Phadia platform. Each sample was analyzed in duplicates.

Statistical analysis

Outlier analyses on the analytical and within-subject level were performed using Cochran's C test whereas outliers on the between-subject level was performed by the Dixon–Reed method [15, 18]. The data distribution of the means for the whole population and each subject separately was assessed by visual evaluation and by the Shapiro–Wilk test. As data followed a normal distribution, the results are presented as mean and range. To evaluate the steady state of the population, a linear regression of the mean value for each blood drawing including all subjects vs. the blood drawing number was performed. All blood drawings were included in the analysis. Subjects were considered in steady state if the 95 % confidence intervals (CI) of the slope of the regression line included zero. The assessment of steady state was conducted for the combined data over the three-day period. Variance homogeneity of within-subject variability was assessed by the Brown–Forsythe test. Pearson's correlation was used to evaluate the correlation between serum tryptase and age.

In accordance with Fraser et al. [18], the analytical variation (CV_A) was estimated from duplicates of every sample, while linear mixed effects models with day and sample as fixed effects and subjects as a random effect were used to calculate the within-subject BV (CV_I) and between-subject BV (CV_G). 95 % CIs for all the BV estimates were calculated according to Roraas et al. [19].

Index of individuality (II) and number of samples required to estimate an individual's homeostatic set-point (n) within ± 10 % with 95 % confidence were calculated with the equations proposed by Fraser et al. [18]: $II = \sqrt{(CV_I^2 + CV_A^2)/CV_G}$ and $n = (z \cdot \sqrt{(CV_I^2 + CV_A^2)/D})^2$, where z is the z-score and D is the desired percentage closeness to the homeostatic set point. The reference change value (RCV) was calculated on ln-transformed data as $RCV = \exp(\pm z \cdot \sqrt{2} \cdot \sigma) - 1$, where $\sigma = \sqrt{\ln(CV_{I+A}^2 + 1)}$ [20].

A $p < 0.05$ was considered significant. For the pair-wise comparisons of day-to-day (3 comparisons) and semidiurnal mean values (10 comparisons), the Bonferroni correction was used to correct for multiple comparisons, and the given p-values are the corrected values. Statistical calculations were performed in STATA 14 (StataCorp) and GraphPad Prism 9.5.1 (GraphPad Software).

Results

Subjects

Thirty-three subjects were included in the study. Their median age was 39 years (range, 22–66 years) and 23 (70 %) of them were women. A total of 183 blood samples were collected during the study period. Two outliers were detected on the analytical level and excluded. In addition, an

outlier was found on the between-subject level, and this subject was excluded from further analysis (6 blood samples). Therefore, 32 subjects and 175 blood samples remained for inclusion. The samples were distributed as follows; all seven blood samples were collected for 12 subjects, six blood samples for one subject (all but the 9 PM blood sample), five blood samples for 14 subjects (all but the 6 PM and 9 PM blood samples), and three blood samples were collected for five subjects (the three 9 AM blood samples). All participants were in steady state, and no heterogeneity of variance was detected.

Biological estimates

The overall mean concentration of serum tryptase in all patients was 4.0 ng/mL (range, 1.3–8.0 ng/mL) (Table 1). For each subject, the mean concentration and range of tryptase are illustrated in Figure 1. No correlation between mean tryptase concentration for each subject and age was found ($r=0.21$, $p=0.251$). None of the subjects met the 120 % plus 2 ng/mL change between any of their measurements.

The overall CV_I was low at 3.7 % (95 % CI 3.0–4.4 %) and noticeably lower than the CV_G , which was 31.5 % (95 % CI 23.1–39.8 %) (Table 1). Accordingly, the II was very low at 0.16. CV_A was 3.4 % (95 % CI 2.9–4.1 %) and only slightly lower than the CV_I . The RCV for an increase in tryptase was 13.3 % at a 95 % level of significance. Only one sample was required

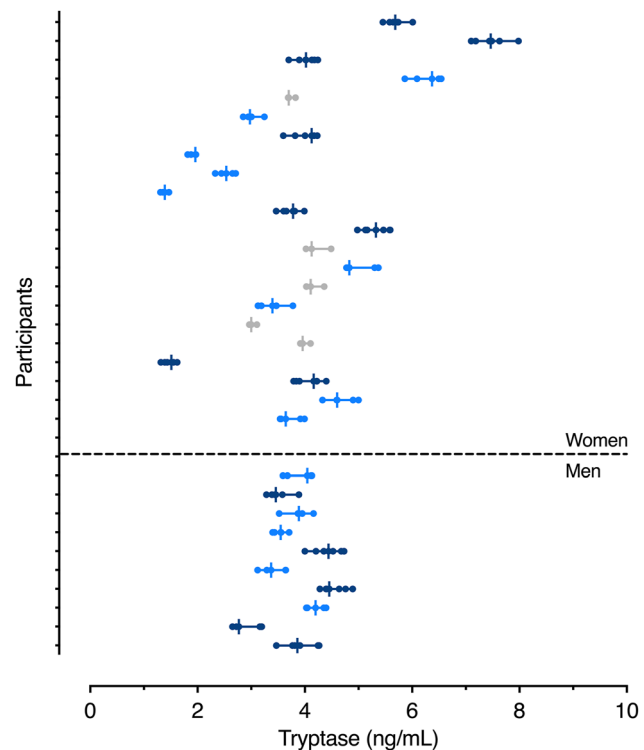


Figure 1: Serum tryptase levels in apparently healthy subjects ($n=32$) ordered according to age with the youngest at the bottom (22 years) and the oldest at the top (66 years) for each sex. Subjects are coloured according to the number of samples contributed by the individual: dark blue, seven or six samples; light blue, five samples; grey, three samples. The coloured vertical lines indicate the mean values and the horizontal lines shows the ranges. The coloured spots illustrate the mean value of duplicates for each measurement.

to provide an estimate of the homeostatic set point within ± 10 % with 95 % confidence.

Table 1: Components of biological variation.

	Tryptase
Number of subjects	32
Number of samples ^a	175
Mean, ng/mL	4.0
Range, ng/mL	1.3–8.0
CV_I , %	3.7 (3.0–4.4)
CV_G , %	31.5 (23.1–39.8)
CV_A , %	3.4 (2.9–4.1)
II^b	0.16
RCV ^c , %	
Increasing	13.3
Decreasing	11.8
Samples required, n^d	1

Values in parentheses are the 95 % confidence intervals. ^aNumber of samples analyzed after exclusion of outliers; ^b II , index of individuality; ^cRCV, reference change value at 95 % significance; ^d n , samples required to estimate homeostatic set point within 10 % with 95 % confidence. CV_I , within-subject coefficient of biological variation; CV_G , between-subject coefficient of biological variation; CV_A , analytical coefficient of variation.

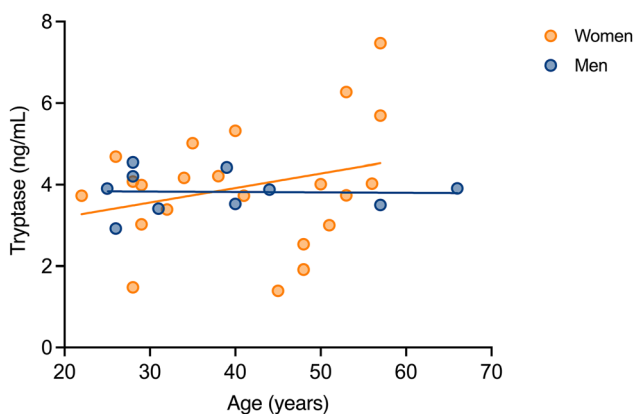
Biological estimates according to sex

Biological estimates were evaluated separately for men ($n=10$) and for women ($n=22$) (Table 2). A greater variability in tryptase concentration was observed in women, although no differences were found in mean concentration or CV_I between sexes. The variability was reflected by the CV_G , which was more than 3-fold higher for women (36.7 %, 95 % CI 26.3–47.1 %) than men (12.9 %, 95 % CI 8.3–17.5 %). Consequently, the II was lower for women (0.14) than for men (0.39). The variability in mean tryptase concentrations in women was primarily found in older women (Figure 2), however, no correlation between mean concentration and age was observed for men ($r=-0.028$, $p=0.938$), women of all ages ($r=0.275$, $p=0.215$), nor women ≥ 50 years ($r=0.628$, $p=0.131$).

Table 2: Components of biological variation according to sex.

	Women	Men
Number of subjects	22	10
Number of samples ^a	115	60
Mean, ng/mL	4.0	3.8
Range, ng/mL	1.3–8.0	2.7–4.9
CV _I , %	3.7 (2.9–4.4)	3.6 (2.1–5.1)
CV _G , %	36.7 (26.3–47.1)	12.9 (8.3–17.5)
II ^b	0.14	0.39
RCV ^c , %		
Increasing	12.9	13.4
Decreasing	11.4	11.8
Samples required, n ^d	1	1

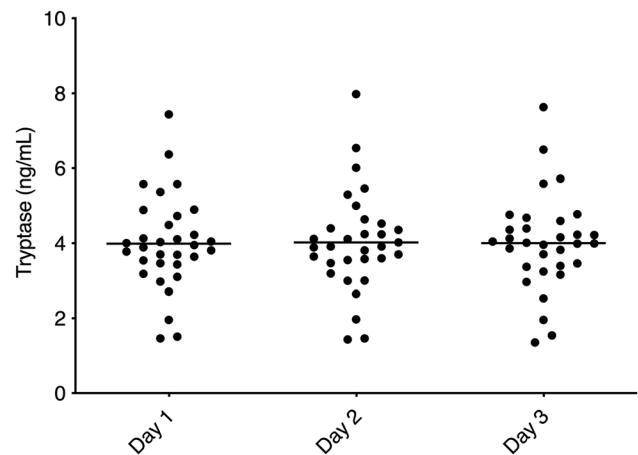
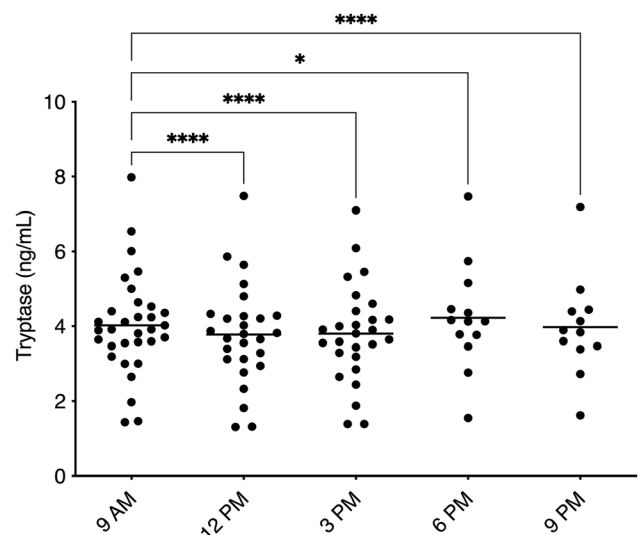
Values in parentheses are the 95 % confidence intervals. ^aNumber of samples analyzed after exclusion of outliers; ^bII, index of individuality; ^cRCV, reference change value at 95 % significance; ^dn, samples required to estimate homeostatic set point within 10 % with 95 % confidence. CV_I, within-subject coefficient of biological variation; CV_G, between-subject coefficient of biological variation; CV_A, analytical coefficient of variation.

**Figure 2:** Relationship between age and serum tryptase mean level for each sex. Women are indicated in orange and men are indicated in blue. The lines show linear regression fits for each sex.

Day-to day and semidiurnal variance

The day-to-day variance components were calculated based on the three 9 AM samples for each subject. No significant difference in serum tryptase concentration was observed between the three days ($p=0.77$, Figure 3). The semidiurnal variance components were calculated based on all the samples collected on day 2 (9 AM, 12 PM, 3 PM, 6 PM, 9 PM). A significant semidiurnal variance was observed between tryptase concentrations at 9 AM and 12 PM, and 3 PM and 9 PM ($p<0.0001$, Figure 4).

The mean concentration of serum tryptase at each time-point is depicted in Table 3 together with the day-to-day and semidiurnal variance components, which were comparable with the overall biological estimates.

**Figure 3:** Day-to-day levels of serum tryptase. Individual results (spots) are depicted. The horizontal lines shows the mean values.**Figure 4:** Within-day levels of serum tryptase from the second day. Individual results (spots) are depicted. The horizontal lines shows the mean values. Significant differences are marked with * $p<0.05$ and **** $p<0.0001$.

Discussion

Despite serum tryptase being used in the diagnosis of several clinical conditions, especially anaphylaxis and mastocytosis, only few studies have addressed the BV of the biomarker. Yet, the presence of individual BV, both long-term as well as over a short period of days or hours, could affect the diagnosis significantly. Therefore, we here presented data on the degree of short-term BV of serum tryptase in a cohort of apparently healthy individuals. We observed no significant day-to-day variation, but found minute, yet significant, variations in the concentration over the day. Furthermore, a

Table 3: Day-to-day and semidiurnal components of biological variation.

	Tryptase
Day-to-day	
Number of subjects	32
Day 1, ng/mL	4.0
Day 2, ng/mL	4.0
Day 3, ng/mL	4.0
CV _I , %	3.9 (3.0–4.7)
CV _G , %	32.2 (23.7–40.8)
Semidiurnal	
Number of subjects	27
12 PM, ng/mL	3.8
03 PM, ng/mL	3.8
06 PM, ng/mL	4.2
09 PM, ng/mL	4.0
CV _I , %	3.4 (2.1–4.7)
CV _G , %	31.4 (23.2–39.6)

Day-to-day and semidiurnal serum tryptase levels are presented as means. Values in parentheses are the 95 % confidence intervals. CV_I, within-subject coefficient of biological variation; CV_G, between-subject coefficient of biological variation.

very low intra-individual variation was identified together with a low RCV. A considerable between-subject variation was found, which was substantial larger for women than men.

The very low within-subject BV observed was in line with a previous study evaluating long-term BV of serum tryptase over a 10-week period (CV_I=5.6 %) [10]. This implicates that the fluctuations of tryptase around the homeostatic set point in steady state are very low and that tryptase is under a tight homeostatic control. This was further confirmed here by a low RCV for an increase in tryptase of only 13.3 %, which shows that for an individual with a 4.0 ng/mL mean tryptase concentration, only an increase of 0.56 ng/mL can be attributed to BV. This clearly uncovers tryptase as a very sensitive biomarker for disease monitoring. For the diagnosis of anaphylaxis, an acute increase in tryptase concentration of 120 % + 2 ng/mL has been recommended [7]. Using an individual with a mean tryptase concentration of 4.0 ng/mL as an example again, this would require an increase in concentration to at least 6.8 ng/mL to confirm the diagnosis, which is far above the BV of tryptase found in the present study. Hence, our data shows that a significant increase in serum tryptase is seen considerable earlier than estimated by the recommended equation. However, it has been suggested in retrospective studies that patients with elevated serum tryptase concentrations owed to diseases such as mastocytosis, atopy, and myeloid disorder have increased basal tryptase variability [8, 21]. Therefore, studies

determining the BV in patients with elevated basal serum tryptase concentration would be highly relevant.

In accordance with previously reported data [10], the CV_G was substantially higher (31.5 %) than the CV_I, indicating that the serum tryptase exhibits great individuality. Because of the low CV_I and moderate CV_G, a low II of 0.16 was observed. An II >1.4 indicates great applicability of conventional reference intervals, whereas an II <0.6 indicates limited applicability [18]. Thus, this finding shows that an individual's normal tryptase level only span a very limited part of the reference interval and that extreme changes for an individual will not necessarily result in values outside the reference range. This supports performing repeated measurements of tryptase as recommended in the diagnosis of anaphylaxis. Furthermore, it emphasizes that using personalized reference intervals could be preferable for serum tryptase [22]. Interestingly, we observed a greater CV_G, and thereby a lower II, for women than for men. Greater variability in tryptase concentrations was primarily seen in women above 50 years. This is surprising, as no sex-dependent differences in serum tryptase levels have previously been observed [23, 24] or were observed in the current study. Potentially, our finding of low variability in men may be due to low statistical power in the male group as only 10 men were included, with eight of these being under the age of 50. However, it has previously been determined that the power of the CV_I estimate is 0.99 when 10 individuals are included who each contributes six samples, two replicates of each sample are analyzed, and the ratio between CV_A and CV_I is around 1 as for men in this study [25]. Hence, we believe that the estimates for both women and men are reliable. Notably, all individuals included in this study had serum tryptase levels within the reference interval as the highest concentration measured was 8 ng/mL [17, 23].

We observed a minute, yet significant, variation in the tryptase level during the day, but no variation from day to day. In accordance with this, Dugas-Breit et al. [11] observed a slight decrease in tryptase concentrations at 2:30 PM and 4 PM compared to baseline samples at 9:30–11 AM in nine control individuals. In the present study, we similarly observed a significant decrease at 12 PM and 3 PM. Nevertheless, the observed changes were minor (4.0 ng/mL at 9 PM, 3.8 ng/mL at 12 and 3 PM), and thus not considered to be clinically relevant, as they are well below the required change for the diagnosis of anaphylaxis.

The very low CV_I found for serum tryptase sets high demands for the analytical performance of the serum tryptase analysis. The desirable analytical performance for CV_A is below or equal to half the CV_I [18]. Even though CV_A was relatively low in our study, it was higher than desired, however, few analytical methods would fulfill a requirement

of a CV_A of maximum 1.85 %. Hence, careful precautions need to be taken to minimize variation as much as possible when measuring serum tryptase. We attempted to minimize pre-analytical variation by using a standardized blood sample collection procedure performed by four technicians only. Moreover, the samples were batch-analyzed in duplicate using a standardized protocol performed by a single laboratory technician. The analysis was run on the commercial Phadia Immunoassay Analyzer using the Immuno-Cap™ sandwich immunoassay. This is the current state-of-the-art set up for serum tryptase analysis, and the analytical performance of the method in our laboratory was better than what has been achieved by others [10].

The strengths of our study were the application of the checklists for BV studies [13–15] and the strict protocol to reduce the impact of pre-analytical variation on the results. Moreover, we included individuals with a wide age range (range 22–66 years with 19 individuals above 34 years), which contrasts with the previous data on BV of tryptase where only younger individuals (23–35 years) were included. However, there were also some limitations to consider. Even though no long-term stability data of tryptase exists, we included blood samples that had been stored at -80°C for four years. Yet, all samples from the same individual were analyzed simultaneously and thus had been stored for the same period of time. Another limitation of the study was that few subjects contributed to the 6 and 9 PM blood draws, resulting in these estimates being less robust. Lastly, the inclusion of subjects was based on clearly defined inclusion and exclusion criteria, which was evaluated based on self-reported information from the subjects. However, no clinical tests were performed to verify the subjects' health status and, hence, we cannot be completely certain that all subjects were actually healthy.

In conclusion, we showed that serum tryptase is a tightly controlled biomarker with a very low within-subject BV, without any significant day-to-day variation, and with only minor semidiurnal variations. In contrast, a considerable between-subject BV was observed, signifying that reference intervals should be cautiously applied with respect to interpreting tryptase levels. Altogether, our data on the BV of tryptase provides valuable information for the clinical interpretation of tryptase levels and underlines its utility as a biomarker for anaphylaxis as well as non-anaphylactic conditions.

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Research ethics: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the Central Denmark Region Committees on Biomedical Research Ethics [1-10-72-452-17].

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflicts of interest.

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Data availability: The raw data can be obtained on request from the corresponding author.

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