Effects of Age, Race, Sex, and Smoking on Prothrombin Fragment 1.2 in a Healthy Population

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Prothrombin fragment 1.2 (F1.2) is a biomarker of thrombin generation during blood coagulation and has diagnostic potential for assessing thrombotic risk and monitoring anticoagulation therapy. We used a monoclonal antibody-based immunoassay for plasma F1.2 to establish a well-defined reference interval and to evaluate the effects of age, race, sex, and smoking status on F1.2 concentrations in a healthy population. Plasma samples and demographic information were obtained from 357 healthy individuals. F1.2 concentrations more closely followed a lognormal than a gaussian frequency distribution. In a multiple linear-regression model in which the logarithms of F1.2 concentrations were regressed on age, race, sex, and smoking status, the significant explanatory variables were age and, to a lesser extent, sex and smoking. A segmented "hockey stick" regression model indicated that F1.2 concentrations and age were unrelated for individuals <44 years old but were positively correlated above that age threshold. The estimated 95% tolerance interval (P = 0.95) for F1.2 in healthy individuals <44 years old (n = 268) was 0.21–2.78 nmol/L. We conclude that age-matched F1.2 reference intervals may be important for studies evaluating the diagnostic utility of F1.2 measurements, and that the clinical relevance of increased thrombin generation during aging warrants further investigation.

Indexing Terms: prothrombin · thrombin · coagulation assays · sex- and age-related effects · smoking · reference interval · enzyme-linked immunosorbent assay

During blood coagulation, prothrombin fragment 1.2 (F1.2) is generated when prothrombin is activated to thrombin by the prothrombinase complex. As a marker of thrombin formation, F1.2 has clinical utility for assessing thrombotic risk and monitoring efficacy of anticoagulant therapy (1–3). For full realization of the clinical potential of F1.2, a well-defined reference range for plasma F1.2 and identification of the critical demographic variables that affect its concentrations are needed.

Mean (±SD) concentrations of plasma F1.2 in healthy populations have been reported to be 1.51 ± 0.68 (n = 31; 1) and 0.67 ± 0.19 nmol/L (n = 95; 3). However, these ranges were determined by using polyclonal antibody-based immunoassays (3, 4) that detect both F1.2 and its potential degradation product F2, and by assuming a gaussian distribution of analyte within the population. We previously reported (2) a mean F1.2 concentration of 1.28 ± 0.70 nmol/L in a small (n = 37) healthy population measured with a monoclonal antibody-based immunoassay specific for F1.2 only (5). Because of the small sample size, rigorous statistical modeling of that population was impractical and, as with the other studies, a gaussian distribution was assumed (2).

As part of the Normative Aging Study, Bauer et al. (6) showed that concentrations of F1.2 plus F2 increase with age in healthy men older than 42 years, which suggests that age alone may be an important demographic variable that affects F1.2. Their study did not include females and, because it included a small number of smokers, no conclusion could be drawn regarding the influence of smoking on F1.2 plus F2.

The purpose of our study was to establish a well-defined reference range for F1.2 only and to evaluate the effect of age, race, sex, and smoking status on F1.2 in a large healthy population.

Materials and Methods

Sample collection. Blood samples were collected from 357 healthy, nonpregnant individuals by trained personnel during the day shift at two geographic sites (Gulf Coast Regional Blood Center, 266 donors; Organon Teknika Corp., Durham, NC, 91 donors) by routine venipuncture into lithium heparin-containing Vacutainer Tubes (14.3 USP units/mL of blood; Becton Dickinson, Rutherford, NJ). Age, race, sex, and smoking status were recorded for each donor. No female donors from the Gulf Coast Regional Blood Center used oral contraceptives; data on oral contraceptive use in female donors from Organon Teknika were not obtained. Plasma was obtained by centrifuging (1000 × g, 15 min, 4 °C) the anticoagulated blood. Plasma was mixed (9/1, by vol) with an EDTA-containing reagent (Organon Teknika’s Sample Treatment Reagent) within 90 min of blood collection and stored frozen at ≤20 °C until assayed.

Plasma F1.2 quantification. F1.2 concentrations were measured by using Organon Teknika’s Thrombostik F1.2 ELISA. Assay specificity is conferred by a monoclonal antibody that binds the F1.2 carboxyl-terminal and a peroxidase-labeled rabbit antibody that tags the F1.2 amino-terminal region (5). For each assay, a calibration curve was generated with use of F1.2 calibrators of 0–10 nmol/L and validated by using low- and high-concentration control samples. Intra- and interassay CVs were typically <10%.

Statistical evaluation. Chi-square goodness-of-fit tests were used to evaluate conformity of F1.2 concentrations to various distributions. Data were analyzed by using

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multiple linear and segmented regression models (7-9). Logarithms of the F1.2 concentrations were used in the regression analyses with significance at \( P < 0.05 \). A power transformation of F1.2 concentrations was used (10) to estimate 95% tolerance intervals for 95% of all future observations from a population similar to the one sampled here (i.e., \( 1 - \alpha = 0.95; P = 0.95 \)). To obtain the interval endpoints in nmol/L units, we detransformed the estimated endpoints on the power-transformed scale.

**Results**

**Characterization of healthy population.** The number of donors and distributions by combinations of race, sex, and smoking status are shown in Figure 1 for each age decile of the healthy population. Mean donor age was 38.9 years (range 17–69 years); 25% of the population was older than 44 years. Only two races (black and Caucasian) were represented. Within the population, 10% were black, 45% were female, and 19% were smokers.

**Characterization of F1.2 distribution.** The frequency distribution of F1.2 in the healthy population is shown in Figure 2. For plotting purposes, only F1.2 values <2.0 nmol/L (96% of values) are presented. Of the values not shown, seven were between 2.0 and 2.8 nmol/L, and nine were >2.8 nmol/L. A similar frequency distribution was obtained when only donors <44 years old were considered (data not shown). The greatest F1.2 concentration for donors <44 years was 9.0 nmol/L; the greatest for the total population was 20.1 nmol/L.

A normal (gaussian) distribution was fit to the F1.2 values (Figure 2) and yielded a chi-square value of 1217 (degrees of freedom = 17), indicative of a poor fit by this model. Because the estimation of a tolerance interval assumes that data follow a gaussian distribution and because untransformed F1.2 concentrations did not meet this assumption, power transformations were applied to the data to identify a model in which application of gaussian statistics would be valid. A logarithmic transformation of the F1.2 concentrations yielded a reasonable fit (chi-square = 69; Figure 2) and was used for subsequent regression analyses. A slight improvement in fit resulted when F1.2 concentrations were raised to a power of −0.7 (chi-square = 20), so we used this transformation for estimating the tolerance interval.

**Influence of demographic variables.** To determine the effects of age, sex, race, smoking status, and collection site on F1.2 concentrations, we fit to the logarithm of F1.2 concentrations several multiple linear-regression models, using these covariates in different combinations. Site was included as a possible variable because blood samples for F1.2 were collected at two geographic locations. A fairly parsimonious model that explained ~11% of the variability of the logarithmic values included terms for age, the square of age, sex, smoking status, site, race, and the interaction of sex and smoking status. Table 1 shows the partial F-statistic as well as the P-value associated with each of these effects. The nonsignificant variables were site, race, and smoking status. The significant explanatory variables by this model were age (\( P = 0.004 \)) and, to a lesser extent, sex and the interaction of sex and smoking status. Correction for the number of models fit, the number of esti
mated effects within each model, and possible higher-order interactions reduced the importance of all but the age effect.

To determine whether there was a particular age at which F1.2 concentrations began to increase within the population, we fit to the data a segmented "hockey stick" model (8, 9), regressing the logarithm of F1.2 concentration on age. The fit of this empirical model is shown in Figure 3, and the results of the regression analysis of variance in Table 2. Partitioning the sums of squares attributable to regression into tests of the importance of model terms, Table 2 shows the following:

- A model with two nonzero slopes and two intercepts does no better at explaining the trend with increasing age than does a model with only one nonzero slope and two intercepts (second slope).
- A model with two intercepts and one slope is significantly better ($P = 0.009$) than a model with one slope and one intercept (break point).
- A model with one slope and one intercept explains significantly ($P = 0.002$) more variability in F1.2 concentrations than does a one-intercept model (linear term).

To summarize, in the best model by this analysis, the logarithm of F1.2 concentrations varied negligibly with age up to a certain age and positively correlated above that age. The estimated age break was 44 years, with upper and lower 95% confidence limits of 31.5 and 53.2 years, respectively.

Based on the 268 individuals <44 years old, the estimated geometric mean of the F1.2 for a similar population is 0.51 nmol/L. An estimated tolerance interval that should contain 95% of future values when testing a similar population 95% of the time is 0.21–2.78 nmol/L.

**Discussion**

Based on plasma F1.2 concentrations in a large (n = 357) population of healthy blood donors measured with a monoclonal antibody-based immunoassay (5) specific for F1.2 only, a well-defined reference interval for plasma F1.2 has been established, and donor age has been identified as a critical demographic variable impacting F1.2 concentrations. Specifically, for individuals <44 years old, a 95% tolerance interval is 0.21–2.78 nmol/L ($P = 0.95$), and for individuals ≥44 years old, F1.2 tends to increase with increasing age. Necessary for statistical validity of a tolerance interval, the frequency distribution must be gaussian, and we ensured that this assumption was met by applying a power transformation to the F1.2 values of the healthy population. One could argue that, based on the significance of the covariate effects on F1.2 (Table 1), separate reference ranges should also be established for sex and smoking categories. However, when corrections for the number of models fit, the number of estimated effects, and the possible higher-order interactions were considered, the need for those additional F1.2 reference ranges could not be justified.

The geometric mean F1.2 concentration of 0.51 nmol/L for healthy individuals <44 years old is, in general, similar to the arithmetic means of 1.51 (1), 1.28 (2), and 0.69 nmol/L (3) previously reported for healthy populations. Direct comparison with these means is complicated because the earlier studies used polyclonal antibody-based immunoassays that measured both F1.2 and its degradation product F2 (1, 3) and (or) applied simple gaussian statistics to the data (1–3). Nonetheless, the general similarity suggests that the product F2 may not be present in large amounts in healthy indi-
individuals. The extent or clinical relevance of F2 formation in unhealthy individuals is unclear.

In this study population, which included both men and women, F1.2 concentrations appear to significantly increase with subject age >44 years. Although the hockey-stick model used is only empirical and other models, e.g., nonlinear, may fit the data equally well, this result substantiates a previous study in which the concentrations of F1.2 plus F2 were shown to increase with age in healthy men >42 years old (6). Our results also suggest that the trend is not sex-specific. Because F1.2 is a biomarker of thrombin generation, the question remains as to whether increased thrombin formation associated with aging reflects subclinical vascular or hemostatic changes of concern in overtly healthy individuals.

F1.2 may be useful as a thrombotic marker, and the mean concentration reported for patients with documented deep venous thrombosis is 6.2 (SD 4.3) nmol/L (2). We measured F1.2 concentrations as great as 9.0 and 20.1 nmol/L in two presumably healthy donors, the former value being from a 17-year-old donor. Although in vitro thrombin generation associated with nonroutine venipuncture of these donors cannot be ruled out, the high F1.2 concentrations may again reflect increased in vivo thrombin generation associated with subclinical vascular or hemostatic conditions of potential concern. Possibly, a single F1.2 reference interval for all ages may be useful for identifying subclinically diseased individuals; however, until the clinical relevance of increased thrombin generation during aging is understood, age-matched F1.2 reference intervals may be important for evaluating the role of F1.2 in assessing thrombotic risk and monitoring anticoagulation therapy.

References

Automated Spectrophotometric Method for Determining Oxidized and Reduced Glutathione in Liver


An enzymatic recycling method has been applied to the measurement of total and oxidized glutathione with a centrifugal analyzer. When the reduced form of glutathione (GSH) was masked with 2-vinylpyridine to measure the oxidized glutathione (GSSG), the time to ensure full derivatization was three times longer than has been reported. The method is quick, simple, accurate, and precise (1.27% for GSH, 3.3% for GSSG intraassay CV; 2.15% for GSH, 5% for GSSG interassay CV), and the automation allows large numbers of samples to be conveniently assayed.

Indexing Terms: centrifugal analyzer · 2-vinylpyridine · enzymatic methods

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Glutathione is a tripeptide widely distributed in nearly all mammalian cells. An important antioxidant, it protects cells against damage from free radicals and toxic endogenous and exogenous compounds either by reacting directly with these toxins or by facilitating the reduction of protein disulfide bridges (1). Concentrations of total glutathione therefore give an indication of the intracellular oxidative state.

One of the antioxidant properties of glutathione is mediated by the enzyme glutathione peroxidase (EC 1.11.1.9). During the detoxification of lipid and other peroxides produced by free radical attack, glutathione peroxidase converts glutathione from a reduced state (GSH) to an oxidized one (GSSG).2 The NADPH-dependent

2 Nonstandard abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; 2-VP, 2-vinylpyridine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); and NEM, N-ethylmaleimide.