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Reference Distributions for Alpha$_2$-Macroglobulin: A Practical, Simple and Clinically Relevant Approach in a Large Cohort.

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In this 11th article in a series, reference values of serum levels alpha$_2$-macroglobulin α_2M) are examined. The study is based on a cohort of 40,420 Caucasian individuals from northern New England that were tested in our laboratory between 1994 and 2000. Measurements were standardized against Certified Reference Material (CRM 470)/Reference Preparation for Proteins in Human Serum (RPPHS) and the results analyzed using a previously described statistical approach. Individuals with unequivocal laboratory evidence of inflammation (C-reactive protein >10 mg/L) were excluded in one leg of the study and included in the other, confirming that α_2M does not respond to acute phase drive in man. Nephrotic syndrome, diabetes mellitus, and chronic liver disease have significant effect on levels of α_2M. Dramatic changes occur during life with males higher from birth to age 12, females thereafter have higher values until the ninth decade. When values were expressed as multiples of the age- and gender-specific median levels, the resulting distributions fitted a log-Gaussian distribution well over a broad range. When patient data are normalized in this manner, the distribution parameters can be used to assign a centile corresponding to an individual's measurement thus simplifying interpretation. J. Clin. Lab. Anal. 18:139–147, 2004.

Key words: reference range; Caucasian; Alpha$_2$-macroglobulin; CRM 470/RPPHS; acute phase response

INTRODUCTION

In vivo pathophysiological events are frequently accompanied by changes in serum protein concentrations. Complex relationships combining up-regulation, consumption, and down-regulation are the rule. However, circulating levels of alpha$_2$-macroglobulin (α_2M) generally remain insulated from the acute phase response in man, but not in laboratory animals (1). Several publications suggest that in unusual circumstances, an acute phase response may affect α_2M levels. The protein's primary recognized function is as a potent inactivator of proteases second only to α_1AT. α_2M is nonselective and will inactivate proteases as diverse as those in snake venom (2). There are two active sites per molecule and even after binding to large molecules, the sites remain active against small substrates. More than 85% of total body mass of α_2M resides within the vascular tree.

α_2M is one of a family of protease inhibitors (alpha$_1$-antitrypsin [α_1AT], C1-inhibitor, alpha$_1$-antichymotrypsin, etc.). Its primary known function is the inhibition of proteinases, by forming irreversible complexes, and the transport of the cytokine interleukin-6 (IL-6) and growth factors. In humans, α_2M synthesis by the liver is not believed to be stimulated by the acute phase response or appreciably by the cytokine IL-6. As the proteinase in highest absolute mass concentration, however, its functional or molar concentration is less than for α_1AT and as such acts as a backup system in the control of proteolytic factors when circulating α_1-antitrypsin levels are low as the result of hypomorphic forms.

Decreased synthesis and concentration can result from progressing hepatic failure. However, in late-stage

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chronic liver disease, levels increase signaling concern for the irreversible hepatorenal syndrome.

Levels of α_2M are increased by estrogen stimulation whether endogenous (pregnancy or hormone shifts during menses) (3) or exogenous (contraceptive medication) (4). Circulating levels increase dramatically in the nephrotic syndrome where the cause appears to involve increased synthesis (5) as well as relative retention by damaged glomerular membranes enabling smaller molecules to escape. As a result of uncertainties about the function of this molecule, testing for α_2M has not attracted much clinical attention. However, new information suggests that a revised perspective on this easily measured protein is in order.

For at least the last two decades, there has been suggestions that α_2M is increased in certain forms of diabetes mellitus, particularly in the presence of microangiopathic complications (6–8). This triad of three recognized situations—high α_2M in the nephrotic syndrome (5), high α_2M in diabetic renal disease (9,10), and association of the nephrotic syndrome with diabetes mellitus (11–13)—thus warrants further investigation using α_2M as a tool for diagnosis and monitoring of diabetic patients. Increased levels repeatedly observed in diabetes may have its genesis in the existence of hyperglycemia (14), which, if fully documented, could provide a new index for glycemic management and life-experience.

Also suspected for years is the increase of α_2M levels in the hepatorenal syndrome, a late phase of cirrhosis associated with vasodilatation of the splanchnic and systemic vascular bed (15,16) leading to decreased renal perfusion and falling glomerular filtration rate. In this circumstance, it perhaps is not unexpected that the balance of synthesis and catabolism of α_2M becomes distorted towards increased concentrations (17). Monitoring α_2M levels might contribute important diagnostic information in the diagnosis of evolving liver and kidney failure considered irreversible up to now.

Genetic polymorphism has been described recently, but there is no available information indicating whether these polymorphisms result in detectable concentration differences. Total absence of the protein has not been described (18), and significantly decreased values rarely occur (19,20). Recent reports have described genetic variants of α_2M (21–24), where gene polymorphism has been associated with tissue deposition of β-amyloid tangles. More recently, several associations between α_2M and dementia have been demonstrated suggesting that α_2M plays a role in the binding of β-amyloid protein somehow preventing fibrillar aggregation and deposition as senile plaques (25). Elevated α_2M levels have been found in Alzheimer's disease (26) and dementia secondary the vascular brain disease (Binswanger's disease) (27). Another group analyzed serum levels of α_2M in 96 elderly Alzheimer's disease patients vs. controls and found no difference (28). When the lipoprotein ε genotypes were studied for α_2M levels, they noted that all three ε3/ε3 homozygous individuals studied had significantly higher levels than any other genotype.

α_2M is regarded as a multifunctional binding protein with high affinity binding sites for zinc, peptides (including cytokines), and a cell surface low-density lipoprotein receptor involved in endocytosis and delivery to lysosomes (29).

The release of myelin basic protein after serious trauma results in an immunogenic material that can lead to immunoreactive demyelination. Binding of myelin by α_2M is now believed to blunt the immunological response to myelin basic protein (30).

The introduction of a reliable reference material (CRM470/RPPHS) (31) and the availability of high quality analytical instruments now provide clinicians with accurate laboratory measurements of α_2M. Providing reliable age- and gender-specific reference data sets the stage for increased clinical utilization of this serum protein in diagnostic medicine.

Presented here is an analysis of α_2M levels from a large Caucasian cohort, measured in one laboratory by the same method and calibrated against CRM 470. During the study the total population (40,420) was analyzed and in a second step the cohort was culled to remove all cases with C-reactive protein (CRP) values over 10 mg/L (28,239). The results demonstrate that, in man, this protein is unaffected by the acute phase response as has previously been suspected from smaller studies. Our final reference range data is based on the entire cohort.

The work described here is a continuation of our previous studies on the immunoglobulins (32), the negative acute phase proteins (33), the positive acute phase proteins (34), serum iron and transferrin saturation (35), and C3 & C4 (36). The individuals forming the cohort are primarily Caucasians who have entered the health care system. A companion paper (37) presents a meta-analysis of published reports of reference ranges covering the time period 1962 to 2001 and places the current findings in that context.

MATERIALS AND METHODS

Serum Protein Reagents and Instrumentation

Antisera was obtained from the same source as in our previous studies and verified in an identical fashion. A clarified and delipidated commercial serum calibrant was used as previously described (32). Values were transferred from CRM 470/RPPHS (obtainable from the Bureau of Community Reference in Brussels,

Belgium (31)). Buffers, other reagents, and the analytic instruments were identical.

SELECTION OF PATIENT RESULTS

Coding of Diagnosis/Symptom Strings

Most samples sent for study by physicians were accompanied by a suspected diagnosis or symptom. Computer processing over 28,000 unique diagnostic strings required that they be classified into 93 categories representing related conditions. For example, the diagnoses of DM, Diabetes, IDDM, SUGAR UP, and over 100 similar entries were clustered into a single category (code 171 representing Diabetes Mellitus, ICD-9 code 250.0). Diagnostic codes for computer processing ranged from 100 (indicating that no diagnosis was provided) to 195 (assigned to diagnoses that were either trivial or could not be deciphered). Code numbers from 101 to 165 were assigned to diseases expected to have a significant impact on serum protein levels (e.g., multiple myeloma, cirrhosis, hepatitis, infection, lung disease, leukemia, renal failure, rheumatic disease, immunodeficiency, etc.). Diagnostic codes 166 through 194 (excepting 170 for pregnancy) contained individuals with conditions expected to have little or no direct effect on serum protein levels (e.g., hypertension, headache, neck pain, neuropathy, syncope, seizures, fatigue, or depression). The lowest code number was given precedence when more than one diagnosis code was present for a given case ensuring that the most significant was used. The diagnostic groups 166 and above (except 170) were designated as the preliminary reference population.

Refinement of the Reference Population

The methodologies for selecting referent individuals and computing the reference ranges have been described in detail elsewhere and above (32–36). In brief, determining which of the diagnostic groups would contribute to the final reference population required the use of a published method which employs symmetric trimming of values prior to calculating means and standard deviations (38). Outliers were identified in this way among the diagnostic group parameters associated with codes not expected to alter serum protein values. A logarithmic transformation of the variance corrected for skewness. The resulting trimmed mean value, ± 1.96 SD for both the multiples of the median (MoM) and the log variance, defined limits of acceptability. Measurements from any diagnostic group falling within the limits were considered reference values; those falling outside were not.

The current dataset is similar to a previous one (33). After exclusion of patient records due to duplication and other reasons (32), 40,420 cases were available for studying α_2M. Of these, 28,239 had CRP levels equal or less than 10 mg/L.

RESULTS

Selection of Diagnostic Groups to Form the Reference Population

Figure 1 shows the median serum levels of α_2M, crudely adjusted for age and gender and plotted against the variance for each of the 85 diagnostic code groups (32) with at least 20 observations, in the same fashion as described in detail previously. Circles represent diagnostic groups coded 166 and above, comprising relatively benign physiologic diagnoses that are not expected to have significant impact on serum protein levels. The squares indicate the diagnostic groups with codes 165 and below, where major protein-effects are expected. The rectangle encompasses the trimmed mean ± 1.96 SD for both the median MoM α_2M levels (horizontal axis) and log variances (vertical axis). Overall, the median and log variances for 64 of the 85 groups lie within the rectangle. Results from individuals within these 64 groups are the referent population for α_2M. Individuals from the 21 diagnostic groups represented by filled circles or squares outside the rectangle were excluded.

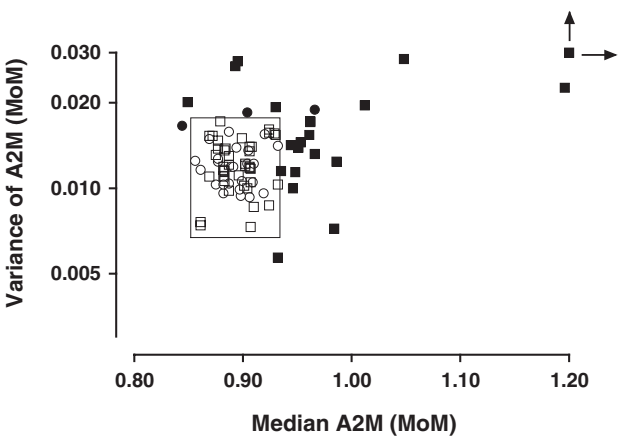

Fig. 1. Scatterplot of α_2M median levels expressed as multiples of the median (MoM) vs. variance for the diagnostic categories after preliminary adjustment for age and gender. Diagnostic codes 100 through 165 are displayed as squares (□, ■), while codes 166 and above are circles (○, ●). The lower the diagnostic group number, the more severe the illness or complaint. The rectangle represents 95% confidence intervals (after trimming) of the median (horizontal axis) and variance (logarithmic vertical axis) for the diagnostic categories 166 and above. Open symbols (○, □) represent those categories whose values comprise the reference population. Closed symbols represent categories not included in reference ranges.

Computation of Age- and Gender-Specific Median Values and Selected Centiles

There were 40,420 referent individuals available to calculate reference ranges for α_2M. Figure 2 shows selected centiles for males (A) and females (B). The logarithmic horizontal axis shows the mean age for each interval while the logarithmic vertical axis shows the median α_2M value (filled circles) along with the 5th and 95th centiles (lower and upper open circles, respectively). The observed centiles for individuals under age 10, 10–30, 30–80, and over 80 years old are based on about 30, 70–600, 500–1200, and less than 200 observations for males, and about 30, 100–1900, 1200–3000, and less than 300 observations for females, respectively. The solid line indicates the fitted median levels in males and the dashed line indicates the fitted medians for females. Results from patients younger than 1 month or older than 85 years of age were not included in the regression analysis.

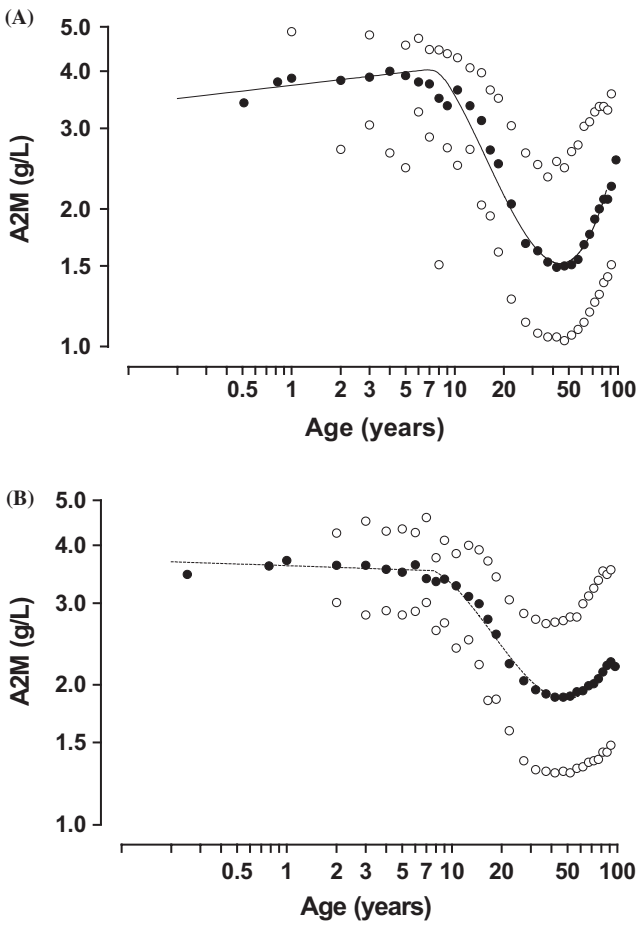

Fig. 2. α_2M centiles and medians vs. age. The individual's age is displayed on the horizontal logarithmic axis vs. the α_2M level on the vertical logarithmic axis. The closed circles (●) represent the observed median level; the lower and upper open circles (○) represent the observed 5th and 95th centiles. The lines represent the predicted median values. **A** (upper) and **B** (lower) show the results for males and females, respectively.

For the first decade of life, males had consistently higher values than females. For both groups, α_2M levels drop significantly during the second and third decades reaching a minimum for both during the fourth decade with male values lower than female. After age 45 values for both sexes climb. Females have, on average, 20% higher median α_2M levels than males through ages 30 to 70. Table 1 contains the gender-specific regression equations for α_2M. A sample computation can be found as a footnote to Table 1.

The distance from the median to the 95th centile and from the median to the 5th centile is approximately equal across the entire age range (Fig. 2A, B). This indicates that the distribution is symmetric after a logarithmic transformation, and that the variance of the distribution does not change appreciably by age.

Fitting the Reference Measurements Expressed in MoM to a Population Distribution

Each individual reference measurement (in mass units) was then converted to a multiple of its age- and gender-specific median value. Figure 3 show a probability plot composed of approximately 2,000 randomly selected normalized referent measurements for α_2M measurements (expressed in MoM). The samples were selected to equally represent each year of age. Measurements fitted the distribution well between the 2nd and

TABLE 1. Regression models and coefficients for median α_2M measurements by age and gender

		Age range (years)		Constant	Coefficients[a]			
Analyte	Sex	From	To	A0	A1	A2	A3	A4
α_2M[b]	Males	0.5	8	0.5710	0.041149	0	0	0
		9	85	−0.7377	4.41513	−4.3447	1.21606	0
	Females	0.5	7	0.5579	−0.01203	0	0	0
		8	85	−0.5570	3.30827	−3.05285	0.81940	0

[a]The general form of the equation is Median = $10^{(A0+A1*\log(age)+A2*\log(age)\wedge 2+A3*\log(age)\wedge 3+A4*\log(age)\wedge 4)}$.
[b]The median α_2M level for a 30 year old female is $10^{(-0.5570+3.30827*\log(30)-3.05285*\log(30)*\log(30)+0.81940*\log(30)*\log(30)*\log(30))}$ or 2.04.

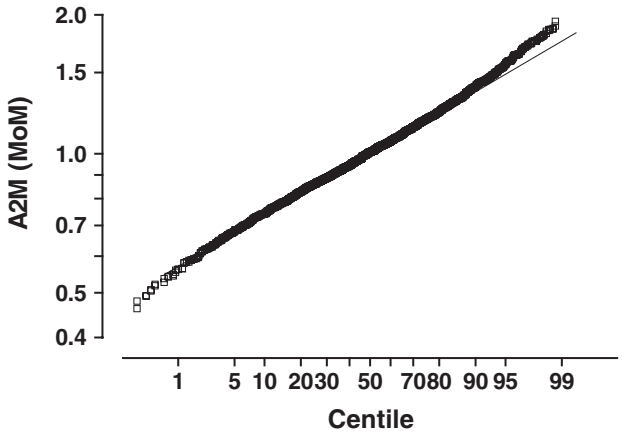

Fig. 3. Probability plots of complement α_2M measurements expressed as multiples of the median (MoM). Approximately 2,000 observed MoM values are plotted vertically on a logarithmic axis and horizontally on the Gaussian centile scale. The latter is based on the rank of the observation. If the points fit a straight line, the distribution is log-Gaussian. The thin line represents the fitted Gaussian means and standard deviations contained in the text.

90th centiles (logarithmic mean and SD of 0.0000 and 0.1048, respectively) and are reassuringly close, up to the 95th centile.

When this analysis was restricted to individuals over 85 years of age, the logarithmic mean was similar to the overall values (log SD of 0.1165). For individuals less than 10 years of age, protein distributions were tighter with a log SD of 0.0606.

Predicting Age- and Gender-Specific Centiles

Within this range of centiles fitted above, patient values can be assigned an age- and gender-specific centile. Table 2 contains the predicted 2.5th, 50th, and 97.5th centiles for selected age and gender categories using the single set of log Gaussian parameters described above. As a further example, consider an α_2M measurement of 3.50 g/L. If this value were to be reported for a 10-year-old male, it would be considered relatively normal (multiple of the median of 0.99 [3.50/3.54] and would be assigned the 48th centile [z-score of -0.04 ((log(0.99)–log(1.00))/0.1048)]. However, if the value were from a 30-year-old female, the interpretation would be quite different (1.72 multiples of the median [3.50/2.04] and would be assigned the 99th centile [z-score of 2.25 ((log(1.72)–log(1.00))/0.1048)].

Verification of Results Over the Course of the Study

In order to check the consistency of results during this study, a median MoM was computed for each of the 20 calendar quarters of the study period. The median MoM level was reasonably consistent over time for α_2M (1.00 ± 0.05). With about 2,000 observations available for each quarter, this variability is highly significant, but likely represents the best control over pre- and intra-analytical assay conditions that can be achieved in routine practice. The median age of patients remained relatively constant at 48 years of age while the percentage of males remained unchanged at 30%.

TABLE 2. Predicted α_2M medians and selected centiles stratified by age and gender

	Males (g/L)			Females (g/L)		
Decimal age (years)	2.5th	50th	95th	2.5th	50th	95th
1.0	2.32	3.72	5.98	2.25	3.61	5.80
4.0	2.46	3.94	6.33	2.21	3.55	5.70
7.0	2.51	4.03	6.47	2.20	3.53	5.66
10.0	2.20	3.54	5.68	2.05	3.29	5.29
14.0	1.74	2.79	4.48	1.79	2.87	4.61
18.0	1.44	2.31	3.71	1.59	2.55	4.09
20.0	1.33	2.14	3.43	1.51	2.42	3.89
30.0	1.04	1.67	2.69	1.27	2.04	3.27
40.0	0.95	1.53	2.46	1.18	1.89	3.04
50.0	0.96	1.54	2.46	1.16	1.86	2.99
60.0	1.02	1.63	2.62	1.19	1.90	3.06
70.0	1.12	1.80	2.89	1.25	2.00	3.21
80.0	1.28	2.05	3.28	1.33	2.14	3.43

DISCUSSION

Proteinase inhibitor levels are becoming more clinically relevant than ever in patient management (5,39–44). The characteristics of serum protein technology measurement at a high level of precision and accuracy (45), a reliable reference material in wide use (31), and the availability of age- and gender-specific reference ranges have set the stage for increased clinical utility of these analytes.

This is the 11th in a series of studies presenting age- and gender-specific reference ranges for α_2M from a single laboratory in a large relatively homogeneous cohort. To address the possibility that α_2M is (33–36) insensitive to the acute phase response, we reanalyzed the data using only those with CRP values less than 10 mg/L.

Direct comparison of MoM values between the two sets of data showed that MoM values were less than 0.7% different and variance was slightly over 4% different; the total group slightly higher than the uninflamed group. Nevertheless, several papers document changes in α_2M levels under special circumstances. Acute pancreatitis, clearly an inflammatory disorder, was shown to lower α_2M levels by 0.3 g/L over controls (46), whereas patients with septicemia demonstrated an

increase of 0.5 g/L (47). The authors proposed that the fall in α_2M was a result of removal of α_2M-protease complexes from the circulation (48). Another group used two-dimensional electrophoresis to demonstrate that α_2M values also decreased after typhoid vaccination. They also showed that values during the steady state in patients with rheumatoid arthritis were lower than in controls (49), again suggesting that α_2M being removed from the circulation in these inflammatory processes seems similar. From a different point of view, one group has shown that α_2M levels correlate inversely with the amount of tissue swelling after immunization for infectious diseases (50). The higher the levels of α_2M, the less local edema, in effect suggesting that the antiproteolytic effect of α_2M reduces local edema. Another group studied a small number of patients with chronic fatigue syndrome (CFS) and found their IL-6 levels and α_2M were elevated (51). In our population, there were 176 cases of CFS. When the values for α_2M were adjusted for age and sex as part of this study, it was found that the mean value was 0.99 MoM, essentially identical to the normal population. α_2M levels in oral disease has attracted work by several researchers. These publications indicate that α_2M levels increase in saliva and bronchial secretions with chronic infection and return towards normal with therapy (52,53). Their conclusion is that the increased concentrations in secretions is the result of transudation through inflamed membranes. Other conditions have been reported to alter α_2M levels. A total of 19 patients with Down syndrome (DS) and depression have been reported to have lower than expected levels of α_2M when compared to 19 cases of DS without depression (54). On the other hand, another group found that 20 children with DS had higher levels than their normal siblings (55). However, these data were not age-adjusted and cannot be taken at face value. Sickle sell disease has also been implicated showing α_2M values 30% higher in patients when compared to normal volunteers (56). Again, these subjects were not adjusted for age or sex; therefore the presented values may not be different.

Concentration differences of α_2M have been observed for some time. For example, 90 diabetics in one study showed mean α_2M levels of 3.13 g/L while a small group of age and sex matched controls was 2.4 g/L (57). In our cohort, there were 225 cases with the diagnosis of diabetes mellitus and CRP levels less than 10 mg/L and 320 cases in the entire group. The two cohorts had similar α_2M distributions and both were outside the reference range for log MoM and log variance.

New evidence suggests that some conditions may alter the serum levels of α_2M as well as nephrosis and hepatic disease. It has been observed that induced hyperglycemia in normal nondiabetic persons results in increased serum concentrations of α_2M (14). Furthermore, evidence is mounting that elevated α_2M levels are seen in diabetics with increased risk of microangiopathic complication, retinopathy, and nephropathy (58–61). An interesting observation has been made suggesting that patients with prostate cancer and bone metastases had extremely low levels of α_2M (62–64). They found that α_2M levels less than 0.5 g/L predicted prostate cancer with bone metastasis. In our population, we identified 45 cases of prostate cancer not specifically designated as having bone metastases, none of whom had α_2M values less than 1 g/L. From a population of 10,976 predominantly Caucasian males, there were two cases with α_2M less than 0.5 g/L (0.18 and 0.45 g/L). Both were 61 years of age, one with a diagnosis of hypokalemia and the other thrombocytosis.

Somewhat anachronistically, α_2M levels are not significantly affected early in chronic liver disease before failure of hepatic protein synthesis. Measurement of α_2M levels has been used to improve the diagnostic assessment of chronic alcoholics with early signs of hepatic fibrosis (17). However, changes are ultimately seen in the hepatorenal syndrome where irreversible liver and kidney failure without histologic changes are the rule. The mechanism is not fully understood but is associated with dilatation of the splanchnic vasculature and coincident constriction of renal vessels (16). Detection of climbing α_2M concentrations could provide early evidence of this complication before major complications develop and perhaps allow effective intervention.

This study is of sufficient size to reliably estimate the effects of age and gender in a Caucasian population and to describe α_2M values harmonized with an accepted reference material and measured by a single protocol. Because the current study is derived from a relatively homogeneous Caucasian population, the findings cannot be applied to other ethnic groups with certainty. Individual usage of tobacco products, over the counter antiinflammatory medications, contraceptives, or hormone supplementation could not be taken into account. Since the half-life of these drugs is short, only carefully designed studies could take their usage into account. As shown in our companion paper (37) and in previous papers (32–35), our data compares favorably with only some of the previous small studies that employed guidelines defining health status (65,66) but did not adjust for age or sex.

Our proposed method of converting laboratory results to multiples of the age- and gender-specific medians (MoM) has several advantages. Conversion permits each analyte to fit a logarithmic Gaussian distribution reasonably well, allowing each MoM level to be assigned a centile. Thus, a laboratory

measurement can not only be reported in mass units but also, through conversion to MoM, the associated centile based on that individual's age and sex. This process greatly simplifies the interpretation of these serum protein values.

CONCLUSION

Routine serum measurements for α_2M performed at a single laboratory in a large homogeneous cohort of Caucasians have been examined to determine the reference distributions. To confirm that α_2M is not affected by inflammation, we have compared the total population including those with an acute phase response with those who have CRP less than 10 mg/L. The conclusion is that α_2M levels are unaffected. As measured by CRP in the world's literature where small cohorts can often have their health status verified (65,66), inflammation shows a remarkable spread of concentration with less than a quarter of the papers being consistent with our data (37). Reference ranges for this protein over a life-time and between the sexes has been described in a large cohort for the first time.

As a result of various studies recently supported by this evaluation, α_2M testing could provide important information about early diabetes and the hepatorenal syndrome. Early diagnosis of both these disorders can provide crucial evidence to inaugurate proper treatment before serious consequences develop.

For this analyte, both age and gender are important covariates and necessitate separate reference ranges. Values clearly differ between the sexes at all ages, except for preadolescents and those over age 85 years of age (see Table 2). Converting the analyte measurements to multiples of the age- and gender-specific medians (MoM) simplifies interpretation and enhances the clinical utility of their measurements. After verification of the appropriateness of medians presented in this study, the predicted values should be considered for use by laboratorians or clinicians making interpretations of α_2M.

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