



# Genotype-corrected reference values for serum angiotensin-converting enzyme

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**ABSTRACT:** The deletion (D)/insertion (I) polymorphism in intron 16 of the angiotensin-converting enzyme (ACE) gene has the greatest impact on serum ACE level in Caucasians of any factor yet discovered. The aim of the present study was to establish new ACE genotype-corrected normal ranges for serum ACE level in a population of central European origin.

After a medical examination, 159 healthy Caucasians volunteered to donate blood for the study. ACE genotypes were assessed by PCR and serum ACE levels were determined using two different kinetic tests.

The distribution of the D/I polymorphism of the ACE gene was in accordance with the Hardy-Weinberg equilibrium. Serum ACE levels and ACE genotypes correlated significantly, with the highest serum ACE levels in subjects with ACE genotype D/D, and the lowest serum ACE levels in subjects with genotype I/I (mean  $\pm$  SD, assay 1: D/D  $59.3 \pm 15.1$  U·L<sup>-1</sup>, D/I  $45.5 \pm 15.2$  U·L<sup>-1</sup>, I/I  $34.8 \pm 13.7$  U·L<sup>-1</sup>; assay 2: D/D  $43.7 \pm 14.1$  U·L<sup>-1</sup>, D/I  $33.7 \pm 12.1$  U·L<sup>-1</sup>, I/I  $25.4 \pm 9.5$  U·L<sup>-1</sup>). Although they gave different absolute values of serum ACE levels, the results of the two test kits correlated significantly.

In conclusion, the present authors recommend the use of new, genotype-specific reference values for serum angiotensin-converting enzyme levels, especially to improve the sensitivity and specificity of tests for angiotensin-converting enzyme in the follow-up of sarcoidosis.

**KEYWORDS:** Angiotensin-converting enzyme, deletion/insertion polymorphism, PCR, reference value, sarcoidosis

Angiotensin I-converting enzyme (ACE; dipeptidyl carboxypeptidase I; Enzyme Commission (EC) 3.4.15.1) is a peptidyl-dipeptide hydrolase belonging to the class of zinc metalloproteases. It has a major role in the metabolism of vasoactive peptides, converting the decapeptide angiotensin I into the active octapeptide angiotensin II, and hydrolysing the vasodilator peptide bradykinin. ACE has been found in many different cell types, including neuronal cells, renal proximal tubular cells, endothelial cells and cells of the monocyte-macrophage system. It is attached to the endothelial surface membrane by an anchor peptide, and can be cleaved, resulting in its release into the blood circulation as a soluble enzyme [1–3].

Serum ACE level is often elevated in patients with untreated active sarcoidosis [4], and spontaneous or corticosteroid-induced remission of sarcoidosis can be heralded by decreasing serum ACE values [5]. Measurement of serum ACE is thus a tool in the monitoring of sarcoidosis. However, although serum ACE concentrations are remarkably stable when measured repeatedly

in a single healthy person, there are large interindividual differences [6]. Thus, the wide reference range of plasma ACE levels hampers the interpretation of ACE values in a clinical setting. CAMBIEN *et al.* [7] found similar serum ACE concentrations in consanguineous family members, suggesting that ACE levels are subject to a strong genetic influence. This was confirmed when a deletion (D)/insertion (I) polymorphism in the ACE gene was found [8], which was later defined precisely by the presence or absence of a 287-bp DNA fragment in intron 16 of the ACE gene. This polymorphism is reported to be associated with  $\leq 28\%$  of the variation in serum ACE level [9], depending on the ethnic background of the population examined. In a study employing a large Nigerian cohort, Cox *et al.* [10] reported that 33% of the variance in ACE serum level was explained by genetic variation, while 19% was explained by residual familial correlations [10]. The remaining 48%, however, was dependent on random individual-specific factors.

Studies of the D/I polymorphism in the ACE gene have shown the D allele to be associated

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with enhanced ACE activity, and the I allele with lower ACE activity [8, 11]. Although the idea of exploiting the D/I polymorphism to improve the clinical value of ACE serum levels is not new, the lack of any definition of reference values according to patient ACE genotype has been noted [8, 12, 13].

The aim of the present study, therefore, was to establish new reference ranges for serum ACE levels in healthy subjects from Germany, in accordance with their ACE D/I genotype.

## MATERIALS AND METHODS

### Subjects

In the present study, 159 healthy adult Caucasians from northern Germany were employed (53 male: mean  $\pm$  SD age  $36.5 \pm 10.7$  yrs, range 18–61 yrs; 106 female: age  $37.7 \pm 11.7$  yrs, 19–64 yrs). Participants were hospital employees who volunteered to donate blood after an examination by the occupational medical service of the Research Centre Borstel (Borstel, Germany). All of the subjects were judged healthy. None of them was on any medication. In particular, subjects were asked about ACE inhibitors. All subjects included in the study denied being on such medication. Informed consent was obtained from all participants.

### DNA extraction and PCR

DNA was extracted from anticoagulated venous blood samples using TRIZOL (Invitrogen, Carlsbad, CA, USA). The D and I alleles were identified on the basis of PCR amplification of the relevant fragments from intron 16 of the ACE gene, followed by size fractionation and visualisation by gel electrophoresis.

The relevant fragment in intron 16 of the ACE gene was amplified according to the method of RIGAT *et al.* [14], as previously described [15]. Briefly, DNA was amplified in a 25- $\mu$ L mixture containing 2.5 mM MgCl<sub>2</sub>, 5 pmol of each primer (primer 1: TGG AGA GCC ACT CCC ATC CTT TCT; primer 2: GAC GTG GCC ATC ACA TTC GTC AGA T; MWG-Biotech AG, Ebersberg, Germany), 0.2 mM of each deoxyribonucleoside triphosphate (Invitrogen), 0.5 U of Taq polymerase (Invitrogen) and 2.5  $\mu$ L 10 $\times$  PCR Buffer (Invitrogen). The PCR ran as follows: 5-min initial denaturation at 95°C; followed by 30 cycles of 30 s at 94°C (denaturation), 30 s at 54°C (annealing) and 1 min at 72°C (extension); followed by a final elongation step at 72°C for 5 min. The presence of the D allele resulted in a 192 bp PCR product, while the I allele resulted in a 479 bp PCR product. Owing to the preferential amplification of the D allele in heterozygous samples, all samples found to have the D/D genotype were included in a second insertion-specific PCR (primer 1: TGG GAC CAC AGC GCC CGC CAC TAC; primer 2: TCG CCA GCC CTC CCA TGC CCA TAA; MWG-Biotech AG). The reaction buffer differed from the previous reaction in that it contained 5% DMSO (Sigma-Aldrich, Munich, Germany) and 1.5 mM MgCl<sub>2</sub>. The PCR conditions were as follows: 5 min initial denaturation at 95°C; followed by 30 cycles of 30 s at 94°C, 45 s at 62°C and 40 s at 72°C; followed by a final elongation step at 72°C for 5 min. The presence of an I allele resulted in a 335 bp PCR product, whereas for samples homozygous for D/D, no products could be detected [16, 17].

Real-time PCR was performed in 20  $\mu$ L iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) containing 5 pmol of each primer, as described above, and 5 ng DNA. PCR was run in a three-step protocol at 95°C, 57°C and 72°C on an iQ Real-Time Detection System (Bio-Rad). After 45 cycles, a melting curve employing a temperature increase of 0.2°C $\cdot$ 10 s<sup>-1</sup> was started.

### Serum ACE measurement

Serum ACE was determined photometrically by two different commercially available kinetic tests, purchased from Bühlmann Laboratories AG (Allschwil, Switzerland; assay 1) and from Trinity Biotech (Bray, Ireland; assay 2). Testing was performed according to the manufacturers' instructions. ACE catalyses the hydrolysis of the synthetic substance N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycylglycine (assay 1) [4, 18] or hippuryl-L-histidyl-L-leucine (assay 2) [19]. This hydrolysis results in a decrease in absorbance at 340 nm, which is measured in both tests.

### Statistical methods

Data are presented as mean  $\pm$  SD. The correlation between ACE genotype and serum ACE level was analysed using the ANOVA test. Correlations were estimated using correlation matrices with Fisher's *r* to *z* as *post hoc* test and Bartlett's test of sphericity. For comparisons of two groups, *p*-values <0.05 were regarded as statistically significant. For multiple comparisons, *p*-values were corrected for the number of groups (0.5/(*n*-1)). Differences between the genotype frequencies within the current study group and the expected frequencies as calculated by the law of Hardy and Weinberg were estimated using Chi-squared tests.

## RESULTS

### Distribution of ACE genotypes

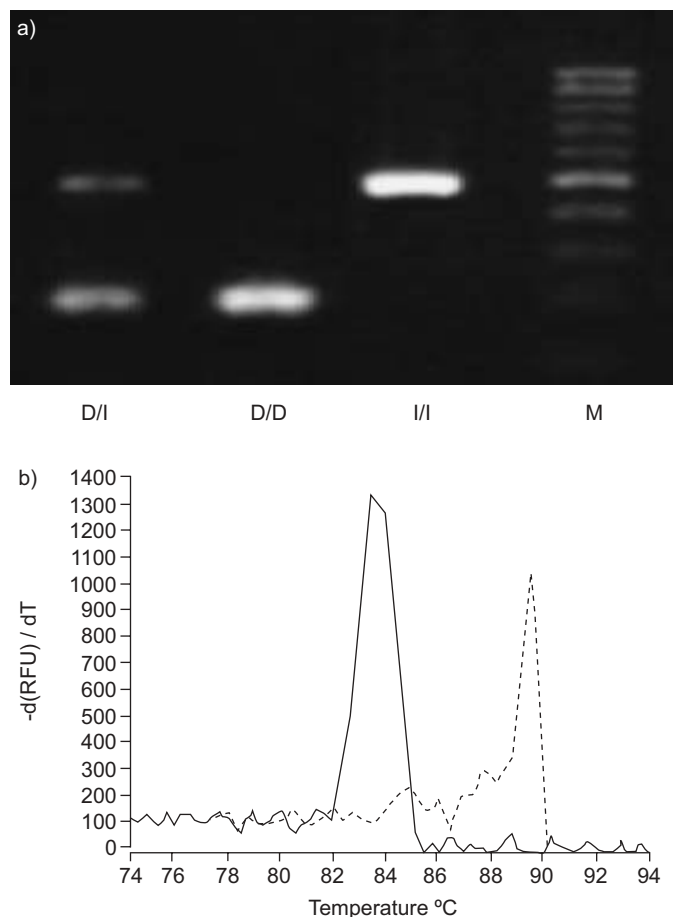
In all cases, initial PCR was successful. Due to the length difference, the I allele band in D/I genotypes is weaker than the D allele band (fig. 1a). Therefore, apparent D/D cases were further investigated employing an insertion-specific primer. This protocol warrants that in every case the correct genotype is determined. The absence of a product in this second PCR reaction confirmed the D/D phenotype. The results of the conventional PCR were in line with results obtained by real-time PCR (fig. 1b).

The homozygous genotype D/D was present in 29.6% of the subjects in the present study, 24.5% were homozygous for the I/I genotype, and 45.9% of subjects had the heterozygous genotype D/I. The distribution of genotypes was in accordance with the Hardy–Weinberg equilibrium (genotype frequency 0.30; 0.46; 0.24 for D/D, D/I and I/I, respectively).

There were no significant differences between genotype frequencies between males and females.

### Serum ACE levels with regard to ACE genotype

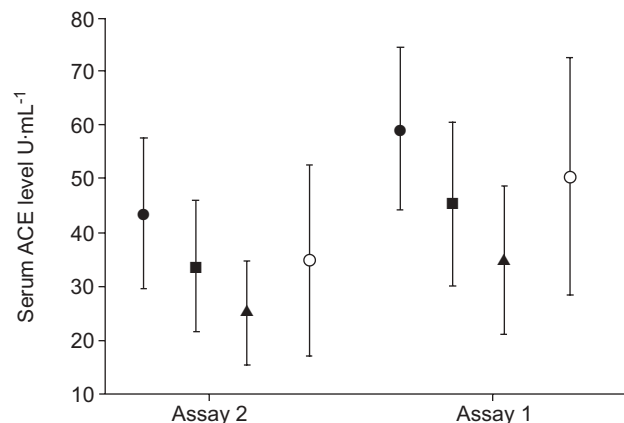
The overall mean serum ACE level detected by assay 2 was  $34.6 \pm 13.8$  U $\cdot$ L<sup>-1</sup> (fig. 2). Based on these results, an upper limit to the normal range of serum ACE levels of 62 U $\cdot$ L<sup>-1</sup> was calculated. This coincides exactly with the value provided by the manufacturer. The overall mean serum ACE level detected by assay 1 was  $47.0 \pm 17.3$  U $\cdot$ L<sup>-1</sup>, leading to an upper limit to



**FIGURE 1.** Examples of angiotensin-converting enzyme genotyping. a) Visualisation of the PCR products on a 2% agarose gel. The presence of a 192-bp PCR product indicates a deletion (D)/D genotype; the presence of a 479-bp PCR product designates the insertion (I)/I genotype. Heterozygotes (D/I) are shown by the presence of both bands. b) Genotyping by melting curve. The length polymorphism, together with an increased GC content, results in an increase in the melting temperature of the I allele. A single peak at 83.5°C (—) indicates a D/D genotype; a single peak at 89.5°C (-----) indicates an I/I genotype. The D/I genotype is indicated by the presence of both peaks (data not shown). M: size markers;  $d(\text{RFU})/dT$ : change in relative fluorescence over temperature.

the normal range value of  $81 \text{ U}\cdot\text{L}^{-1}$ , which is close to the value provided by the company ( $79 \text{ U}\cdot\text{L}^{-1}$ ). This high reproducibility of the upper limits indicate that the individuals selected in the present cohort are suitable for the study, because they resemble the cohorts originally used to establish the thresholds.

Using assay 2: the mean serum ACE in subjects with genotype D/D was  $43.7 \pm 14.1 \text{ U}\cdot\text{L}^{-1}$ ; in those with genotype D/I, mean serum ACE was  $33.7 \pm 12.1 \text{ U}\cdot\text{L}^{-1}$ ; and in those with genotype I/I, mean serum ACE was  $25.4 \pm 9.5 \text{ U}\cdot\text{L}^{-1}$ . Using assay 1: the mean serum ACE in subjects with genotype D/D was  $59.3 \pm 15.1 \text{ U}\cdot\text{L}^{-1}$ ; in those with genotype D/I, mean serum ACE was  $45.5 \pm 15.2 \text{ U}\cdot\text{L}^{-1}$ ; and in those with genotype I/I, mean serum ACE was  $34.8 \pm 13.7 \text{ U}\cdot\text{L}^{-1}$ . Regardless of the test kit used, there was a highly significant correlation between ACE genotype and serum level, as determined by ANOVA ( $p < 0.0001$ ). Distribution analysis of the ACE concentrations



**FIGURE 2.** Mean serum angiotensin-converting enzyme (ACE) levels of patients with different ACE genotypes. Serum ACE levels were determined colorimetrically using two different assays as described in the Materials and Methods section. Serum ACE levels and the ACE genotypes showed a significant correlation ( $p < 0.0001$ ). Data are presented as mean  $\pm$  SD. ●: deletion (D)/D; ■: D/insertion (I); ▲: I/I; ○: the ACE level of the entire cohort without regard to genotype.

revealed a Gaussian distribution of the values in all three genotypes. Owing to the normal distribution of the ACE concentrations and the Hardy–Weinberg equilibrium of the genotypes, no additional statistical analysis, as applied for genotypes not following the Hardy–Weinberg equilibrium or whose frequencies are not normally distributed, were performed.

Table 1 shows the 95% confidence intervals (mean  $\pm 1.96\text{SD}$ ) for the serum ACE levels for each genotype, suggesting new clinical reference intervals for serum ACE levels. When using assay 2, an upper reference level of  $71 \text{ U}\cdot\text{L}^{-1}$  is suggested for individuals with genotype D/D, an upper limit of  $57 \text{ U}\cdot\text{L}^{-1}$  for those with genotype D/I and an upper reference value of  $44 \text{ U}\cdot\text{L}^{-1}$  for those with genotype I/I. For assay 1, upper reference levels of  $89 \text{ U}\cdot\text{L}^{-1}$ ,  $75 \text{ U}\cdot\text{L}^{-1}$  and  $62 \text{ U}\cdot\text{L}^{-1}$  are suggested for individuals with genotype D/D, D/I, and I/I, respectively. These values differ significantly from those delineated from the entire cohort, not corrected for genotypes (assay 1:  $79 \text{ U}\cdot\text{L}^{-1}$ ; assay 2:  $62 \text{ U}\cdot\text{L}^{-1}$ ).

#### Influence of age and sex on serum ACE levels

There was no correlation between serum ACE levels and age within the present study group, regardless of the test employed.

Interestingly, the results of assay 1 suggested that males had significantly higher serum ACE levels than females ( $51.4 \text{ U}\cdot\text{L}^{-1}$  versus  $45 \text{ U}\cdot\text{L}^{-1}$ , respectively;  $p < 0.05$ ). This difference was not shown by assay 2 ( $36.6 \text{ U}\cdot\text{L}^{-1}$  versus  $33.8 \text{ U}\cdot\text{L}^{-1}$ , respectively; not significant). When the results were broken down by genotype, a male–female difference was apparent only in individuals with the D/I genotype. Interestingly, this difference was shown up by both assay 1 (male:  $52.5 \text{ U}\cdot\text{L}^{-1}$ ; female:  $41.6 \text{ U}\cdot\text{L}^{-1}$ ;  $p < 0.005$ ) and assay 2 (male:  $38.6 \text{ U}\cdot\text{L}^{-1}$ ; female:  $30.8 \text{ U}\cdot\text{L}^{-1}$ ;  $p < 0.005$ ).

**TABLE 1** Genotype-corrected reference values for serum angiotensin-converting enzyme (ACE)

	Subjects n	ACE levels U·L <sup>-1</sup>	
		Assay 1	Assay 2
<b>Manufacturers' reference values</b>		79	62
<b>Genotype</b>			
All individuals	159	12–82	7–62
D/D	47	30–89	16–71
D/I	73	16–75	10–57
I/I	39	8–62	7–44

Data are presented as 95% confidence intervals (arithmetic mean ± 1.96sd), unless otherwise stated. D: deletion; I: insertion.

**Correlation of ACE serum levels as detected by the two kits employed**

Serum ACE levels in 159 samples were measured using both ACE test kits. There was a significant correlation between the ACE serum levels detected by the two methods (p<0.0001, r=0.8; fig. 3).

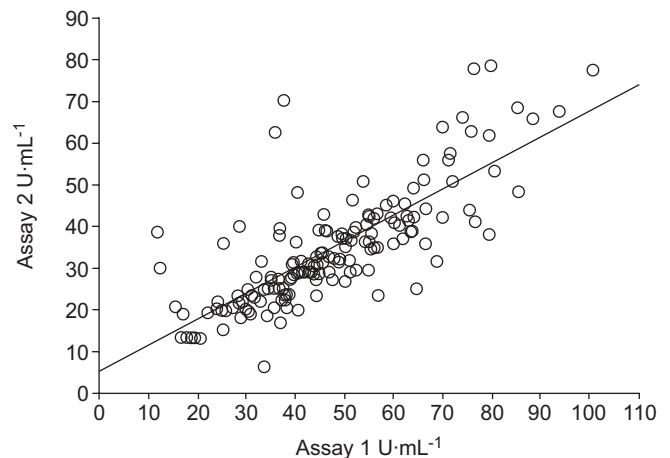
**Clinical relevance**

Figure 4 shows examples of the serum ACE course of sarcoidosis patients of each genotype. Most informative is patient A (genotype I/I; fig. 1a). After successful therapy, the patient's ACE level at day 1 was normal. At day 129, however, the patient suffered from deterioration requiring corticosteroid therapy. Nevertheless, serum ACE remained in the uncorrected normal range. However, use of the corrected range for the I/I genotype disclosed that the patient's serum ACE level was in fact elevated during the deterioration. After reaching a remission, a normal value was again observed. Thus, a change in granuloma burden did not disclose its pathogenetic relevance when the uncorrected reference values were used.

For patient B (genotype D/I; fig. 4b), genotype-corrected values are not relevant. At day 280, the patient presented with increased serum ACE activity, whereas at all other days serum ACE level was within the normal range, regardless of whether the values were genotype-corrected. However, genotype correction lead to a lower threshold and the exclusion of any homozygotes results in higher specificity and sensitivity of the test. On the basis of uncorrected values, patient C (genotype D/D; fig. 4c) appears to have increased serum ACE activity at every time-point, despite being in remission at day 1. Based on genotype-corrected values, serum ACE at day 1 is within the normal range, whereas at the other time-points this patient has elevated levels, which is in accordance with progressing disease. The disease relapse was obvious 6 weeks later.

**DISCUSSION**

In the present study, a close correlation between the D/I polymorphism in the ACE gene and serum ACE levels in a healthy Caucasian population from Germany is described, as has been described previously in populations of other origins [8, 11]. The highest serum levels of ACE were found in subjects

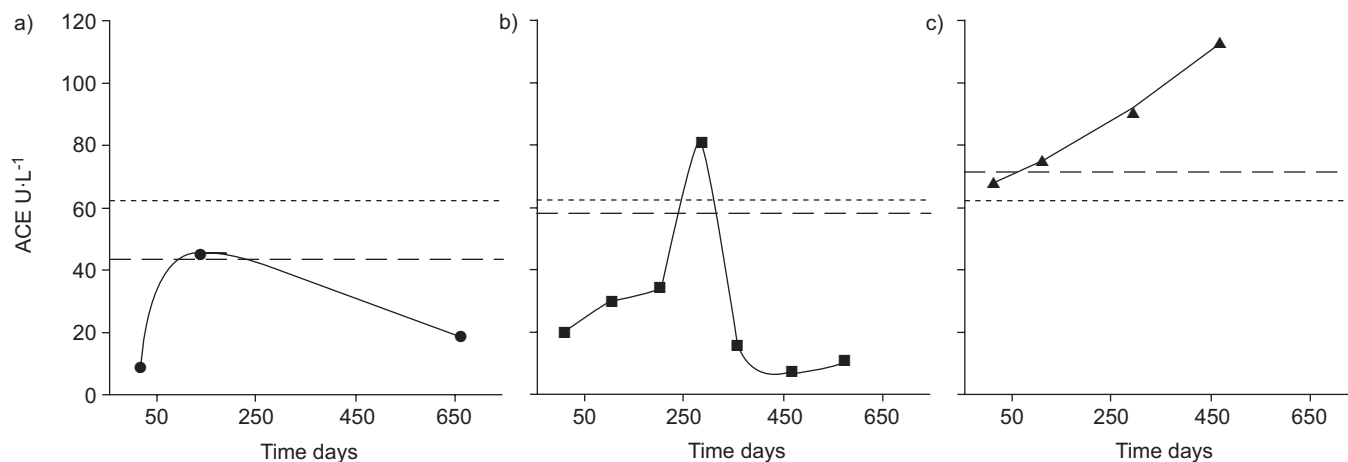


**FIGURE 3.** Correlation of serum angiotensin-converting enzyme (ACE) levels as detected by the two kits employed and measured according to the manufacturers' instructions. The correlation of serum ACE levels detected by the two different tests is shown (r=0.8, p<0.0001).

with the homozygous genotype D/D, followed by those with the heterozygous genotype D/I. The lowest serum ACE levels were found in subjects with the homozygous genotype I/I. Based on these data, new reference intervals for serum ACE levels are suggested (table 1), taking into account the patient's genotype.

The D/I polymorphism is located in a nontranscribed part (intron 16) of the ACE gene. In a study employing a large cohort from Nigeria, ZHU *et al.* [20] demonstrated that, rather than the D/I polymorphism itself, a single nucleotide polymorphism (SNP) in exon 17 (A/G) and an additional SNP in the upstream untranslated region (5'-UTR; A/T) are responsible for the variation in ACE serum activity [20]. Again in a Nigerian cohort, Cox *et al.* [10] established a set of nine haplotypes defined by four different SNPs covering 80% of the observations in their cohort. Regarding serum ACE levels, five of these haplotypes disclose a very small confidence interval. Therefore, the possibility of using haplotypes, rather than the D/I polymorphism alone, was discussed in the literature. However, the situation in Caucasians is completely different. Within populations of African origin, a great variety of polymorphisms is found, and serum ACE levels are not linked to the D/I polymorphism [21]. In Caucasians, however, there are (grossly) eight different haplotypes, with only three haplotypes covering 90% of the observations [22]. In addition, these haplotypes exhibit a strong linkage disequilibrium. Therefore, analysing D/I polymorphisms instead of multiple SNP analysis is a simple and reliable approximation and is thought to mirror the functional SNP in exon 17. In addition, the simple method allows its usage in routine work. However, the new reference intervals suggested in this work cannot simply be adapted to other non-Caucasian populations.

As the current data indicate, using reference ranges as given by manufacturers without regarding genotypes might lead to misinterpretations of ACE serum levels. Normal ACE serum levels might be regarded, falsely, as elevated in subjects with ACE genotype D/D, whereas elevated ACE serum levels in



**FIGURE 4.** Clinical course of serum angiotensin-converting enzyme (ACE) activity from sarcoidosis patients. The graphs depict serum ACE levels, as measured by assay 2 in three patients from the current authors' outpatient clinic with genotypes: a) deletion (D)/D; b) D/insertion (I); and c) I/I. - - -: uncorrected limit for the test employed. - · - · -: corrected limits for D/D ( $71 \text{ U}\cdot\text{L}^{-1}$ ), D/I ( $57 \text{ U}\cdot\text{L}^{-1}$ ) and I/I ( $44 \text{ U}\cdot\text{L}^{-1}$ ) genotypes for assay 2.

patients with ACE genotype I/I might falsely be interpreted as normal. The currently employed upper normal limits suggested by the manufacturers appear to be appropriate only for subjects with the heterozygous ACE genotype D/I (assay 1:  $68 \text{ U}\cdot\text{L}^{-1}$ ; assay 2:  $52 \text{ U}\cdot\text{L}^{-1}$ ). In addition, because average serum ACE levels in a heterogeneous population depend on the frequency of the three genotypes within this population, the uncorrected reference value should be adjusted according to the frequency distribution of the D/I genotypes. Using the genotype-related reference levels as suggested in the current study, a more precise interpretation of ACE serum levels will be possible. Moreover, because reference levels are established for a specific genetically defined subpopulation, the genetic composition of the population as a whole does not influence the reference values, which may thus be applied to all populations of Caucasian origin.

Although statistical analysis of the current data indicates that serum ACE levels are also dependent upon sex (at least using assay 1), this difference was not apparent in the D/D and I/I genotypes. Within the D/I group, the difference between male and female is  $11 \text{ U}\cdot\text{L}^{-1}$  (assay 1) or  $8 \text{ U}\cdot\text{L}^{-1}$  (assay 2). It is possible that reference values for males and females, at least in the D/I genotype, might be helpful. Because females were overrepresented in the present study cohort, a bias towards lower reference values within the D/I genotype cannot be excluded. However, correcting the reference values for sex ratio revealed a difference of  $<3 \text{ U}\cdot\text{L}^{-1}$ , which might be negligible in clinical routine.

As demonstrated by the clinical examples in figure 4, the most important changes will be seen in the homozygote genotypes, because the differences between corrected and uncorrected upper limits are the greatest. In D/I genotypes, the upper levels are not greatly different. Interestingly, in patient A (fig. 4a), the genotype-corrected values indicated an increased ACE level compatible with the worsened clinical situation of this patient, whereas the uncorrected value did not reveal this course. From early indications, the present authors believe the genotype-corrected limits to be more informative.

The close relation between the ACE genotype and serum ACE levels has previously been described [8, 12, 23]. Owing to the use of different nonstandardised methods for ACE detection, the ACE serum levels described in those studies cannot be compared with each other, and reference ranges need to be established for every individual test [13]. In the current study, two of the most commonly employed laboratory tests for serum ACE level were used. Even though a significant correlation was found between the serum ACE levels detected by the tests, the absolute values differed. Therefore, the ACE reference intervals presented in the current study can only be applied if one of the two assays tested is used, on individuals of Caucasian origin. Laboratories using other systems and chemistries need to establish their own reference population and genotype-corrected reference values.

So far, interpretation of serum angiotensin-converting enzyme levels in the clinical monitoring of sarcoidosis patients has been difficult. In contrast to earlier reports showing an elevation of serum angiotensin-converting enzyme levels in sarcoidosis [4], it has long been known that patients with active and progressive sarcoidosis can also have normal or only slightly elevated serum angiotensin-converting enzyme levels [24–26]. In addition, the prognostic value of serum angiotensin-converting enzyme in sarcoid patients has been disputed. While some investigators suggest that the angiotensin-converting enzyme serum levels are reduced during therapy and increased in parallel to a relapse [5, 24], others could not find prognostic information in the serum angiotensin-converting enzyme levels of patients with sarcoidosis [25–29]. Thus, an individual approach to the interpretation of the serum angiotensin-converting enzyme level has been suggested, taking into account the trend of the individual patient's serum angiotensin-converting enzyme level rather than its absolute value [24]. Using genotype-corrected reference values, as introduced in the current study, will yield a higher sensitivity and specificity for the interpretation of serum angiotensin-converting enzyme levels. The current examples illustrate that the increase in sensitivity using genotype-corrected reference levels allows a differentiation of

“increased” and normal serum angiotensin-converting enzyme levels that is paralleled by the clinical course of the patient. However, the exact gain in sensitivity and specificity still needs to be established in large follow-up studies of patients with sarcoidosis. Based on the current data, the present authors recommend the use of new, genotype-specific reference values for serum angiotensin-converting enzyme levels. Those laboratories which take care of patients of Caucasian origin and use one of the two kits tested can employ these genotype-corrected normal ranges.

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