

## A Novel Albumin Gene Mutation (R222I) in Familial Dysalbuminemic Hyperthyroxinemia

Nadia Schoenmakers,\* Carla Moran,\* Irene Campi, Maura Agostini, Olivia Bacon, Odelia Rajanayagam, John Schwabe, Sonia Bradbury, Timothy Barrett, Frank Geoghegan, Maralyn Druce, Paolo Beck-Peccoz, Angela O'Toole, Penelope Clark, Michelle Bignell, Greta Lyons, David Halsall, Mark Gurnell, and Krishna Chatterjee

University of Cambridge Metabolic Research Laboratories (N.S., C.M., M.A., O.R., G.L., M.G., K.C.), Wellcome Trust-Medical Research Council Institute of Metabolic Science, Addenbrooke's Hospital, Cambridge CB2 0QQ, United Kingdom; Institute of Endocrine Sciences (I.C., P.B.-P.), University of Milan, 20132 Milan, Italy; Department of Biochemistry (J.S.), University of Leicester, Leicester LE1 7RH, United Kingdom; Departments of Paediatrics (T.B.) and Clinical Chemistry (M.B.), Birmingham Childrens Hospital, Birmingham B15 2TT, United Kingdom; Departments of Endocrinology (M.D.), St Bartholomew's Hospital, London E1 4NS, United Kingdom; Department of Clinical Biochemistry (F.G.), Ealing Hospital, London UB1 3EU, United Kingdom; Department of Clinical Biochemistry (A.O., P.C.), Selly Oak Hospital, Birmingham B29 6JD, United Kingdom; and Department of Clinical Biochemistry (O.B., S.B., D.H.), Addenbrooke's Hospital, Cambridge CB2 2OO, United Kingdom

**Context:** Familial dysalbuminemic hyperthyroxinemia, characterized by abnormal circulating albumin with increased  $T_4$  affinity, causes artefactual elevation of free  $T_4$  concentrations in euthyroid individuals.

**Objective:** Four unrelated index cases with discordant thyroid function tests in different assay platforms were investigated.

**Design and Results:** Laboratory biochemical assessment, radiolabeled  $T_4$  binding studies, and *ALB* sequencing were undertaken.  $^{125}I$ - $T_4$  binding to both serum and albumin in affected individuals was markedly increased, comparable with known familial dysalbuminemic hyperthyroxinemia cases. Sequencing showed heterozygosity for a novel *ALB* mutation (arginine to isoleucine at codon 222, R222I) in all four cases and segregation of the genetic defect with abnormal biochemical phenotype in one family. Molecular modeling indicates that arginine 222 is located within a high-affinity  $T_4$  binding site in albumin, with substitution by isoleucine, which has a smaller side chain predicted to reduce steric hindrance, thereby facilitating  $T_4$  and  $rT_3$  binding. When tested in current immunoassays, serum free  $T_4$  values from R222I heterozygotes were more measurably abnormal in one-step vs two-step assay architectures. Total  $rT_3$  measurements were also abnormally elevated.

**Conclusions:** A novel mutation (R222I) in the *ALB* gene mediates dominantly inherited dysalbuminemic hyperthyroxinemia. Susceptibility of current free  $T_4$  immunoassays to interference by this mutant albumin suggests likely future identification of individuals with this variant binding protein. (*J Clin Endocrinol Metab* 99: E1381–E1386, 2014)

**F**amilial dysalbuminemic hyperthyroxinemia (FDH), the most common heritable cause of elevated total  $T_4$  levels in euthyroid subjects, has an estimated prevalence of

1 in 10 000 individuals (1). Consistent with its dominant inheritance, the disorder is associated with heterozygous albumin (*ALB*) gene defects, generating mutant proteins

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\* N.S. and C.M. contributed equally to this work.

Abbreviations: ALB, albumin; FDH, familial dysalbuminemic hyperthyroxinemia; FT4, free  $T_4$ ; RR, reference range; TBG,  $T_4$  binding globulin.

with enhanced  $T_4$  binding affinity. An arginine-to-histidine mutation at residue 218 (R218H) was first described (2, 3) and is the most common causal variant in Caucasians but also recognized in Hispanic/Puerto Rican (4) and Chinese (5) cases. Substitution of proline for arginine at the same codon (R218P), resulting in markedly elevated  $T_4$  concentrations, has been described in Japanese and Swiss subjects (6, 7). A third albumin mutation (L66P), identified in a Thai kindred, is associated with predominant elevation of  $T_3$  concentrations (8).

Here we describe a novel, heterozygous *ALB* defect, with substitution of isoleucine for arginine at codon 222 (R222I) in three African (Somali) subjects and one East European (Croatian) family, identified on the basis of discrepant thyroid function tests, with hyperthyroxinemia. Enhanced  $T_4$  binding to this albumin variant correlates with molecular modeling showing that this amino acid change likely reduces steric hindrance within its high-affinity  $T_4$  binding pocket. Elevated free  $T_4$  measurements in most commonly used immunoassay platforms suggests that additional cases harboring this novel FDH variant will be identified.

## Patients and Methods

### Methods

All investigations were part of an ethically approved protocol and/or clinically indicated, being undertaken with the consent from patients and/or next of kin.

### Biochemical measurements

Thyroid hormones [free  $T_4$  (FT4) and free  $T_3$ ] and TSH were measured using automated immunoassay systems (Advia Centaur; Siemens; Wallac DELFIA Ultr; PerkinElmer; Access; Beckman-Coulter; Elecsys; Roche Diagnostics; Architect; Abbott Diagnostics).  $T_4$  binding globulin (TBG) was measured by immunoassay (Siemens Immulite). Equilibrium dialysis FT4 was measured by RIA (Quest Diagnostics). Total  $T_3$  and  $rT_3$  were measured in deproteinized samples by separation using C18 column chromatography followed by electrospray mass spectrometry or ( $rT_3$  in a subset of cases) by competitive RIA (Quest Diagnostics).

### Radiolabeled $T_4$ binding studies and gel electrophoresis

Serum binding of  $^{125}\text{I}$ - $T_4$  was assayed with excess cold  $T_4$  to saturate binding sites on TBG as described previously (9); inclusion of cold  $rT_3$  in this assay enabled comparison of its binding with R218H and R222I albumin mutants.  $^{125}\text{I}$ - $T_4$  binding to serum proteins was analyzed by gel electrophoresis (Mayo Medical Laboratories) as described previously (10).

### Albumin gene sequencing

Exons of the human albumin gene were PCR amplified from genomic DNA using specific primers (listed in Supplemental Material) and analyzed by Sanger sequencing.

## Molecular modeling

The R222I mutant albumin was modeled (Pymol) using previously described wild-type albumin (1bm0) albumin- $T_4$  (1hk1), R218H FDH mutant albumin- $T_4$  (1hk2), and R218P FDH mutant albumin- $T_4$  (1hk3) crystal structures (11), selecting the rotamer with the fewest clashes.

## Results

### Clinical and biochemical features

Proband 1 was a 2.5-year-old, Somalian boy (P1), investigated for low weight, was found to have elevated FT4 but unsuppressed TSH (Table 1). His mother and two siblings exhibited similarly abnormal thyroid function tests [mother: FT4 36.9 pmol/L, (reference range) [RR] 10–24), TSH 1.57 mU/L (0.5–5.0); sibling 1: FT4 30.9 pmol/L (RR 11–22), TSH 2.01 mU/L (RR 0.4–3.5); sibling 2: FT4 48.5 (12–25), TSH 3 mU/L (RR 0.4–3.5)]. Proband 2 was an unrelated 41-year-old Somali male (P2) and was referred with a similar biochemistry (Table 1). Proband 3 was a 20-year-old Somali female (P3), investigated for fatigue and weight gain, and showed hyperthyroxinemia with nonsuppressed TSH (Table 1). Proband 4 was a 22-year-old Caucasian female (P4) from Croatia, investigated for asthenia and anxiety, and was found to have hyperthyroxinemia with nonsuppressed TSH. Her sibling and father exhibited similar thyroid function tests [father: FT4 41.2 pmol/L (RR 10–22), TSH 3.2 mU/L (RR 0.28–4.3); sibling: FT4 33.5 pmol/L (RR 10–22), TSH 3.0 mU/L (RR 0.28–4.3)].

Although local testing in all probands showed markedly raised FT4 concentrations, FT4 measurements using the two-step DELFIA method were quite discordant, being near normal; furthermore, FT4 measured by equilibrium dialysis was normal (Table 1). These observations suggested analytical interference with FT4 measurement, with diagnostic possibilities including abnormal circulating thyroid hormone binding proteins. Although total  $T_4$  was raised in each proband, the TBG levels were normal (Table 1). Hence, an albumin protein abnormality was considered. Serum binding of  $^{125}\text{I}$ - $T_4$  in each proband was markedly raised (Table 1), comparable with values (28%–44%) in sera from known FDH cases, harboring the R218H albumin mutation. Gel electrophoresis of serum from an affected individual identified excess  $^{125}\text{I}$ - $T_4$  binding to albumin [Figure 1A, panel (ii)]. The abnormal electrophoretic profile was similar to the pattern of  $^{125}\text{I}$ - $T_4$  binding in serum from a known FDH case, harboring the R218H albumin mutation [Figure 1A, panel (i)].

### Molecular genetic studies

*ALB* sequencing of probands (P1–P4) revealed heterozygosity for a single-nucleotide substitution (AGA

**Table 1.** Biochemical Measurements in Index Cases

	Proband 1	Proband 2	Proband 3	Proband 4
TSH, mU/L	1.89	1.4	1.71	3.6
Platform	Immulite 2000	Roche Elecsys	Roche Elecsys	Roche Elecsys
Reference range <sup>a</sup>	0.3–4.0	0.27–4.2	0.3–4.0	0.28–4.3
FT4, pmol/L	<b>35.3</b>	<b>50.9</b>	<b>39.3</b>	<b>37</b>
Platform	Immulite 2000	Roche Elecsys	Roche Elecsys	Roche Elecsys
Reference range <sup>a</sup>	12–25	12–22	9–20	10–22
FT4, pmol/L	<b>21.8</b>	<b>22</b>	<b>20.7</b>	16
Platform	DELFLIA	DELFLIA	DELFLIA	DELFLIA
Reference range	9–20	9–20	9–20	9–20
FT4 by equilibrium dialysis, ng/dL	2.2	ND	2.1	1.7
Platform	Quest	ND	Quest	Quest
Reference range <sup>a</sup>	0.8–2.7	ND	1.0–2.4	0.8–2.7
Total T <sub>4</sub> , nmol/L	<b>303</b>	<b>273</b>	<b>275</b>	<b>204</b>
Platform	DELFLIA	DELFLIA	DELFLIA	DELFLIA
Reference range	69–141	69–141	69–141	69–141
TBG, μg/mL	20.3	20.5	20.6	16.5
Platform	Immulite	Cisbio	Immulite	Immulite
Reference range	14–31	11.3–28.9	14–31	14–31
Radiolabeled T <sub>4</sub> binding to serum	37%	Increased <sup>b</sup>	38%	49%
Reference range	<20%	<20%	<20%	<20%

Numbers in bold denote that they are outside the reference range. Abbreviation: ND, not done.

<sup>a</sup> Varying reference data for the same assay platform reflect differing normal ranges used by local laboratories.

<sup>b</sup> Exact percentage binding unavailable.

to ATA), corresponding to an arginine to isoleucine change at codon 222 in the predicted protein sequence, with no other coding region changes. The mutation is not present in 100 control DNA samples and normal genome data sets (dbSNP, 1000 Genomes) including more than 2000 African-American alleles (Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, WA, <http://evs.gs.washington.edu/EVS/>, May 11, 2013). Genotyping for single-nucleotide polymorphisms around *ALB* indicates that the Somali cases share an extended haplotype, suggesting common ancestry, whereas the mutation occurs on a different haplotype background in Caucasian proband 4 (Supplemental Figure 1).

The mother and siblings of P1, with abnormal thyroid function tests, were also heterozygous for this nucleotide change. The *ALB* mutation cosegregated with phenotype in family members of P4, being present in individuals (father and brother) with elevated FT4 results and serum <sup>125</sup>I-T<sub>4</sub> binding and absent in her unaffected mother with normal FT4 concentrations and radiolabeled hormone binding (Supplemental Figure 2).

#### FT4, T<sub>3</sub>, and rT<sub>3</sub> measurements in affected cases

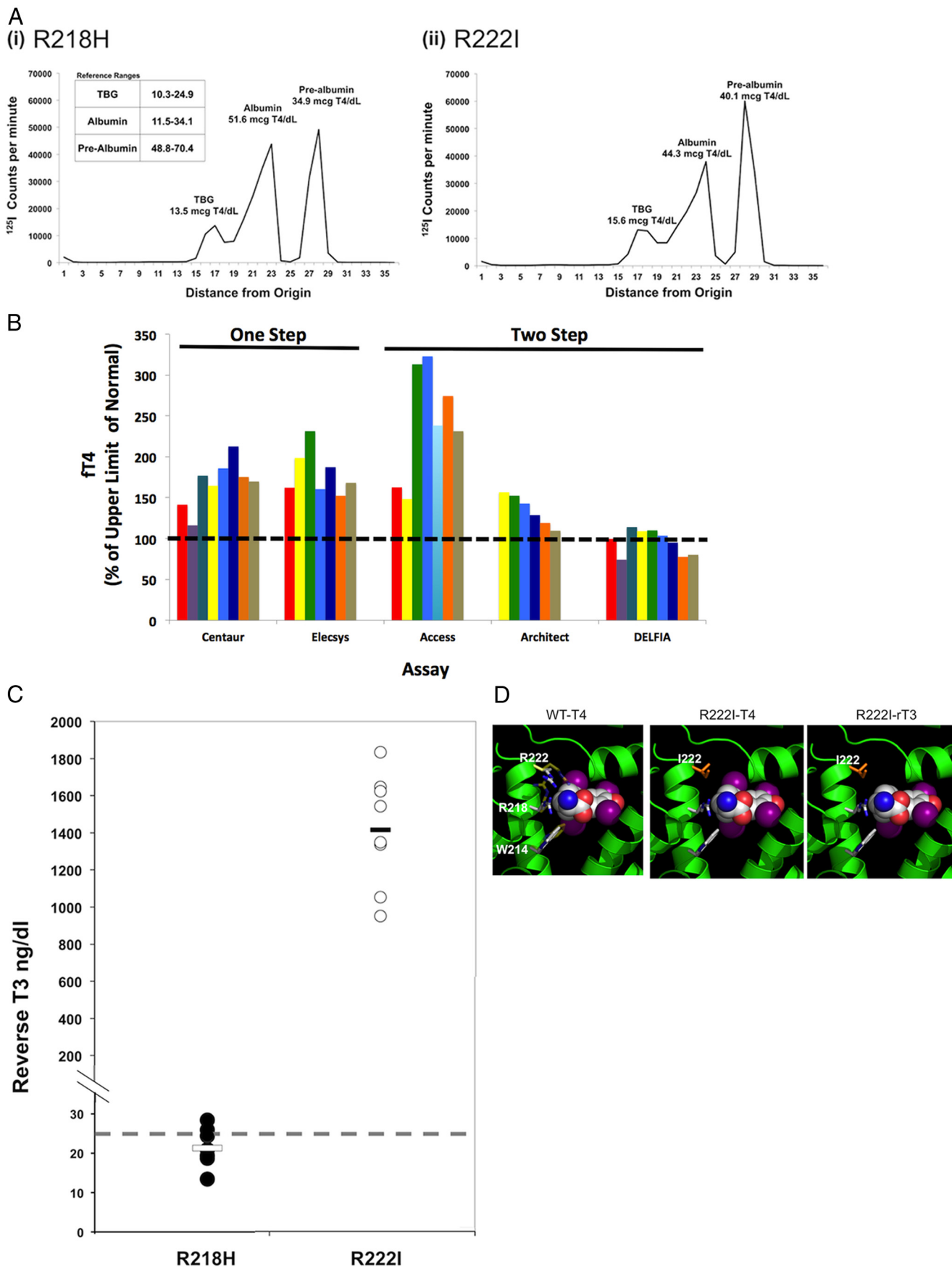
The index cases were identified on the basis of discordant FT4 results using one-step (Roche Elecsys or Siemens Immulite) hormone assays. To investigate the performance of commonly used assay platforms, FT4 concentrations were measured using sera from *ALB* R222I heterozygotes in different two-step [(DELFLIA Ultra (PerkinElmer), Architect (Abbot Diagnostics), Access

(Beckman Coulter)] and one-step [Advia Centaur (Siemens Medical Diagnostics); Elecsys E170 (Roche)] immunoassays (Figure 1B). Affected individuals exhibited a similar pattern, with FT4 measurements being more elevated in one-step (Centaur, Elecsys) than two-step (Architect, Delfia) platforms; exceptionally, FT4 values were most markedly raised with the two-step Beckman Access method.

We assayed total T<sub>3</sub> and rT<sub>3</sub> by tandem mass spectrometry using *ALB* R222I heterozygote sera and compared concentrations with R218H *ALB* FDH cases. Total T<sub>3</sub> concentrations were slightly raised in two R222I *ALB* cases but normal in all other subjects with either variant albumin (Supplemental Figure 3). In contrast, rT<sub>3</sub> concentrations were markedly elevated in *ALB* R222I sera, being 40- to 70-fold elevated, but were normal or only marginally raised (1.1-fold) in R218H *ALB* FDH cases (Figure 1C). Such elevation was also seen when rT<sub>3</sub> was measured by immunoassay in sera from R222I FDH cases (rT<sub>3</sub> > 2 ng/mL, normal range 0.11–0.32 ng/mL). rT<sub>3</sub> displaced <sup>125</sup>I-T<sub>4</sub> binding to R222I *ALB* sera much more readily than in control or R218H FDH cases (Supplemental Figure 4).

#### Molecular modeling of R222I mutant albumin

In the T<sub>4</sub>-albumin crystal structure, T<sub>4</sub> interacts with side chains of three residues (R218, W214, and R222) within a high-affinity binding site. Comparison with an unoccupied protein structure shows that T<sub>4</sub> binding requires significant rearrangement of these three side chains



**Figure 1.** Biochemical studies in FDH cases and molecular modeling of albumin mutation. A, Electrophoregrams showing binding of  $^{125}\text{I}$ -T<sub>4</sub> to serum proteins in serum containing an albumin mutation (R218H) known to confer FDH [left panel, (i)] and an individual with hyperthyroxinemia

(Figure 1D, left panel). Substitution of arginine at codon 222 by isoleucine reduces steric hindrance, enhancing  $T_4$  binding (Figure 1D, middle panel). Iodines in the inner ring of  $T_4$  are in close contact with side chains of R222 and W214. Superimposition of  $rT_3$  (Figure 1D, right panel) with  $T_4$ , reveals that the absence of an inner ring iodine would provide more space in the pocket, with both the isoleucine 222 and tryptophan 214 imposing less steric hindrance; in contrast, substitutions at R218 are not predicted to influence  $rT_3$  binding.

## Discussion

Six individuals from three unrelated families of East African and three subjects of Caucasian East European origin were found to have euthyroid hyperthyroxinemia and nonsuppressed TSH concentrations, with assay-dependent discordant FT4 measurements suggesting analytical interference. Normal circulating TBG concentrations together with increased radiolabeled  $^{125}I$ - $T_4$  binding to serum or albumin from these cases suggested an *ALB* abnormality. Affected individuals are heterozygous for a missense *ALB* mutation (R222I); in one kindred, in which family members were available, heterozygosity for this *ALB* mutation segregates with both abnormal thyroid biochemical and  $^{125}I$ - $T_4$  binding phenotypes.

The high-affinity binding site for  $T_4$  in albumin contains three residues (R218, R222, and W214) whose side chains undergo marked displacement to accommodate  $T_4$  binding (11). Consistent with this structural observation, substitution of histidine or proline with smaller side chains for arginine 218 likely reduces steric hindrance, explaining enhanced  $T_4$  binding of these mutant proteins (11, 12). Likewise, modeling predicts that substitution of isoleucine for arginine 222, as occurs in our cases, also reduces steric hindrance. Indeed, an artificial albumin mutant (R222M), with a methionine residue with smaller side chain replacing R222, exhibits increased  $T_4$  binding (13).

Our results suggest that, in general, one-step FT4 immunoassay methods are more susceptible to interference by R222I FDH sera than two-step designs. This pattern resembles differential susceptibility of such assays with R218H FDH sera (14), presumably reflecting the interaction of labeled  $T_4$  analogs with albumin in one-step assays,

which does not occur in two-step or back-titration methods. It has been suggested that incubation buffer composition in the Beckman Access assay promotes  $T_4$ -albumin interaction, making this two-step method unexpectedly susceptible to interference (15).

Total  $T_3$  concentrations were raised in two R222I FDH sera and normal in R218H FDH cases; this finding is in accord with  $T_3$  concentrations being raised in only 12% of R218H FDH (3). In contrast, total  $rT_3$  concentrations were uniformly and more strikingly elevated in R222I sera than R218H FDH cases. Previously, raised  $rT_3$  was documented in 50% of R218H FDH cases (8) and the R218H mutant albumin binds  $rT_3$  with increased affinity (13). Because total hormone levels likely reflect hormone interaction with albumin in subjects with otherwise normal TH binding proteins, we hypothesized that  $rT_3$  binding to R222I mutant albumin is enhanced, and competition assays with radiolabeled  $T_4$  confirmed this. Structural modeling suggests a basis for this, with the absence of an inner ring iodine in  $rT_3$ , likely to further diminish steric hindrance from side chains of residues (Ile 222, Trp 214) which are in closest proximity to the inner ring iodines. The biochemical pattern of raised  $T_4$ , normal  $T_3$ , and elevated  $rT_3$  concentrations in R222I FDH resembles that seen in patients after amiodarone exposure (16), raising the possibility that this genetic form of FDH might be confused with other clinical diagnostic possibilities.

In summary, we have identified a novel, heterozygous *ALB* mutation (R222I) in subjects of both East African and Caucasian Eastern European origin. R222I heterozygote sera exhibit a biochemical profile of elevated FT4 concentrations in many current immunoassay platforms, suggesting that this genetic cause of dysalbuminemic hyperthyroxinemia will be readily identified, perhaps in other populations.

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**Figure 1 Continued.** and elevated radiolabeled  $T_4$  binding to serum containing an R222I albumin mutation [right panel, (ii)]. B, FT4 measured by various one-step or two-step immunoassays in sera from different cases containing the R222I mutant albumin protein. C,  $rT_3$  measured by liquid chromatography and tandem mass spectrometry in sera from R218H and R222I mutation cases. D, Crystallographic modeling of  $T_4$ , bound to the high-affinity  $T_4$ -binding site in subdomain IIA of the albumin molecule, illustrating the steric constraints imposed on  $T_4$  binding. The left panel is a composite, showing the positions (in yellow) of the side chains of W214, R218, and R222 in the albumin structure not bound to  $T_4$ , superimposed on these displaced side chains (white) in the structure of albumin bound to  $T_4$ . When R222 is replaced by isoleucine (middle panel, in orange), the shorter side chain presents less steric hindrance to  $T_4$  binding.  $rT_3$  binding to R222I mutant albumin is also likely to be enhanced (right panel) because the loss of the inner iodine will further relieve steric hindrance with side chains of residues at positions 222 and 214.

Address all correspondence and requests for reprints to: V Krishna K. Chatterjee, Metabolic Research Laboratories, Wellcome Trust–Medical Research Council Institute of Metabolic Science, University of Cambridge, Level 4, Box 289, Addenbrooke's Hospital, Cambridge CB2 0QQ, United Kingdom. E-mail: kkc1@medschl.cam.ac.uk.

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