Apolipoprotein A-IV polymorphism and its effect on plasma lipid and apolipoprotein concentrations

Peter de Knijff,* Maryvonne Rosseneu,† Ulrike Beisiegel, Walter de Keersgieter,† Rune R. Frants,†† and Louis M. Havekes***

Gaubius Institute TNO,* Leiden, The Netherlands; Department of Clinical Chemistry, St. Jan General Hospital,† Brugge, Belgium; University Hospital Eppendorf,** Hamburg, FRG; and Department of Human Genetics, State University Leiden,††Leiden, The Netherlands

Abstract Recently, we determined the apolipoprotein E (apoE) phenotype distribution in 2,000 randomly selected 35-year-old male individuals by slab gel isoelectric focusing of delipidated plasma samples, followed by immunoblotting using anti-apoE antiserum. These blots have been successfully re-used for immunovisualization of apoA-IV isoelectric focusing patterns. In a population sample of 1,393 individuals, four distinct apoA-IV isoforms were detected, encoded by the alleles A-IV*0, A-IV*1, A-IV*2, and A-IV*3 with gene frequencies of 0.002, 0.901, 0.079, and 0.018, respectively. The mean of plasma cholesterol, triglyceride, apoB and E levels did not differ significantly among the different apoA-IV phenotype groups. For these lipoprotein parameters, less than 0.1% of the total phenotypic variance could be accounted for by the APOA-IV gene locus. Our results did not show any effect of apoA-IV polymorphism on plasma apoA-I levels nor could we find any correlation between plasma levels of apoA-I and apoA-IV within the different apoA-IV phenotype groups. The plasma level of apoA-IV in subjects bearing the A-IV*3 allele is significantly lower than in subjects without the A-IV*3 allele (5 mg/dl versus 14 mg/dl). We therefore conclude that, in contrast to the apoE polymorphism, the polymorphism at the APOA-IV locus does not influence any of the levels of the lipoprotein parameters considered except apoA-IV. —**de Knijff, P., M. Rosseneu, U. Beisiegel, W. de Keersgieter, R. R. Frants, and L. M. Havekes.** Apolipoprotein A-IV polymorphism and its effect on plasma lipid and apolipoprotein concentrations. *J. Lipid Res.* 1988. **29:** 1621–1627.

Supplementary key words apolipoprotein A-IV isoforms • relative allele frequencies • average allelic effect • genetic variance

The presence of apolipoprotein A-IV (apoA-IV) in man has been reported by several groups of investigators (1–3). ApoA-IV is a glycoprotein with a molecular weight of 46,000 and is synthesized in enterocytes of the small intestine during fat absorption. It is secreted into the lymph as a protein constituent of chylomicrons (4,5). Entering the plasma, chylomicrons lose most of their apoA-IV (6). In plasma, apoA-IV is associated with both chylomicrons and high density lipoproteins (HDL). However, after ultracentrifugation the major part of plasma apoA-IV is found in the lipoprotein-free fraction of density > 1.25 g/ml (7).

Differences in structure or composition between the lipoprotein-associated and lipoprotein-free form of apoA-IV have not been observed (7). The plasma concentration of apoA-IV is found to be diminished in abetalipoproteinemia and increased after fat feeding (8,9). This suggests that apoA-IV might be involved in the synthesis and/or secretion of chylomicrons via the lymph into the plasma. At present, several lines of evidence have been reported that apoA-IV is involved in the activation of the enzyme lecithin:cholesterol acyltransferase (10) and in the transport of cholesterol from the periphery back to the liver (11).

Menzel, Kovary, and Assmann (12) and Utermann et al. (13) reported that human apoA-IV can be separated by isoelectric focusing into two major isoforms: apoA-IV-1 and apoA-IV-2, apoA-IV-1 being a single charge unit more acidic than apoA-IV-2. Menzel et al. (12) postulate that this heterogeneity of apoA-IV is the result of two codominant APOA-IV alleles at a single genetic locus. Later, genetic polymorphism of human apoA-IV has also been detected by Kamboh and Ferrell (14) and by Bisgaier, Lee, and Glickman (15) by means of one-dimensional isoelectric focusing of plasma samples followed by immunoblotting. Using this technique, both groups of investigators (14,15) also found two common major apoA-IV isoforms. Using the nomenclature introduced by Menzel et al. (12), the frequency of the APOA-IV*1 allele is about 0.90, whereas the frequency of the APOA-IV*2 allele is about 0.10. Besides these common apoA-IV isoforms, less frequent apoA-IV isoforms have also been reported. Menzel et al. (12) found an apoA-IV isoform slightly more basic than apoA-IV-2 (apoA-IV-Münster). A similar iso-

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins.

form was found by Bisgaier et al. (15). The latter authors also found an apoA-IV isoform slightly more acidic than apoA-IV-1.

The gene for apoA-IV is located on chromosome 11 and is closely linked to the genes coding for apoA-I and C-III (16). Recently, Elshourbagy et al. (17) reported on the structure and expression of the APOA-IV gene in man. To our knowledge, variations in the APOA-IV gene giving rise to the known apoA-IV polymorphism have not, as yet, been described.

Although apoA-IV has been suggested as being involved in lipoprotein metabolism (8–11), at present an association of polymorphism of apoA-IV with plasma lipid and apolipoprotein levels has not been reported. In this study we report the apoA-IV phenotype distribution in a population sample of 1,393 35-year-old male individuals, randomly selected from the Dutch population. For this apoA-IV phenotyping, we used the nitrocellulose blots that have been used earlier for apoE phenotyping in the same population (18). Concomitant measurements of plasma cholesterol, triglycerides, apoB, E, A-I, and A-IV concentrations enabled us to evaluate whether or not these lipid parameters are influenced by apoA-IV polymorphism.

MATERIALS AND METHODS

Collection of plasma samples

Plasma (EDTA) from nonfasting subjects was obtained by venapuncture and stored at −20°C until the assays were performed.

Population sample

In an earlier screening study for apoE polymorphism (18), 2,000 35-year-old males were randomly selected from three different areas in the Netherlands. Differences between these areas with respect to apoE phenotype distribution and plasma lipid and apolipoprotein levels were not observed. All the subjects from two of these areas (n = 1393) were used for the screening in the present study.

ApoE phenotyping

ApoE phenotyping was performed using a recently developed rapid micromethod which is based on isoelectric focusing (pH range 5 to 7) of delipidated plasma samples, followed by immunoblotting on nitrocellulose filter using a polyclonal anti-apoE antiserum (19).

ApoA-IV phenotyping

ApoA-IV phenotyping was performed exactly as carried out for apoE, except that polyclonal anti-apoA-IV antiserum was used as first antibody. For the population screening we were able to use pre-existing apoE immunoblots (pH range 5–7). The filters (stored in the dark for more than 1 year) were incubated in buffer A (0.15 M NaCl, 10 mM Tris-HCl, 0.05 vol.% Tween 20, pH 7.4) in the presence of 0.1% (by volume) of rabbit anti-apoA-IV antiserum. After 1 hr of incubation at room temperature, the filters were washed (twice, 15 min) in buffer A and further incubated in buffer A, containing 0.1% (by volume) of goat anti-rabbit IgG conjugated to horseradish peroxidase. After 1 hr of incubation at room temperature, the filters were washed (twice, 15 min) and developed as previously described (19).

Determination of levels of cholesterol, triglycerides, apoB, E, A-I and A-IV

Plasma cholesterol and triglycerides were measured enzymatically using Boehringer testkits (cholesterol CHOD-PAP and triglyceride GPO-PAP, respectively). The interassay coefficients of variation of both assays were 2%. ApoB concentrations were measured by an immunophelometric assay (INA) as described by Rosseneu et al. (20). Plasma apoE (21), A-I (22), and A-IV (23) levels were measured by enzyme-linked immunosorbent assays (ELISA). The interassay coefficients of variation for the determination of apoB, E, A-I, and A-IV were between 7 and 8%.

Statistical analyses

Allele frequencies were estimated using the gene-counting method. Linkage dysequilibrium between alleles in the APOA-IV and E locus was evaluated by chi-square analysis. Differences in mean lipid and apolipoprotein levels between apoA-IV phenotypic groups were evaluated by either parametric (one-way analysis of variance) or nonparametric (Kruskal-Wallis) statistical tests. In pairs, differences between apoA-IV phenotypic groups were estimated by using either the procedure of Scheffe (parametric test) and the Mann-Whitney U-Wilcoxon rank sum (nonparametric) test or by Student's *t*-test for unpaired samples. The variance of cholesterol, triglyceride, apoB, and apoE concentrations attributable to genotypic differences was estimated exactly according to the method of Sing and Davignon (24).

RESULTS

Method of apoA-IV phenotyping

Recently, we determined the apoE phenotype distribution in a population of 2,000 randomly selected 35-year-old males by using isoelectric focusing (pH 5 to 7) of plasma followed by immunoblotting (18). In order to avoid a second isoelectric focusing of a large number of plasmas from the same population, we re-used the immunoblots that arose from that study for apoA-IV phenotyping. Visualization of the apoA-IV polymor-

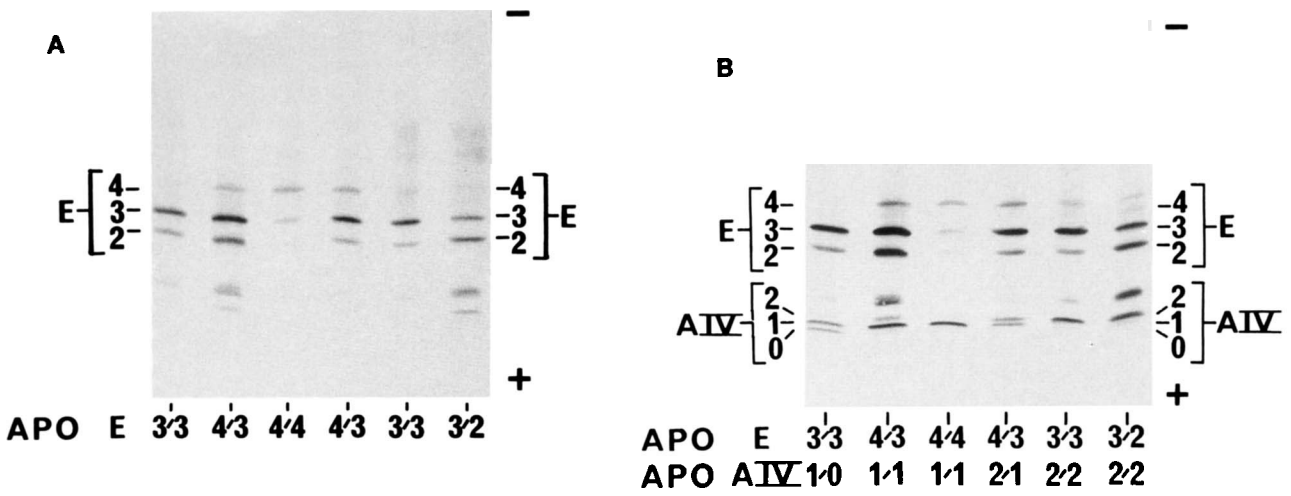

Fig. 1. Isoelectric focusing of two identical series of six different plasma samples followed by immunoblotting. After isoelectric focusing in a pH range of 5 to 7 the whole blot was immunovisualized using anti-apoE antiserum as first antibody (A). Thereafter, one half of the blot was further immuno-stained using anti-apoA-IV antiserum as first antibody (B).

phism on the pre-existing apoE immunoblots was possible as no immunocrossreactivity between apoA-IV, A-I, and E was found (results not shown).

Crossreactivity between apoA-I and apoA-IV, in particular, would be problematic since some minor apoA-I isoforms have overlapping pI values with apoA-IV.

Fig. 1 shows two identical series of six different plasma samples focused on one gel (pH 5–7). After blotting on nitrocellulose, the whole filter was immuno-visualized using anti-apoE antiserum as first antibodies. After drying, one half of the blot (representing one of the two series) was further immuno-stained using anti-apoA-IV antiserum as first antibody (Fig. 1B). Since the pI value of apoA-IV isoforms lies around pH 5 (7), it was questionable whether isoelectric focusing with a pH range of 5 to 7 was suitable for apoA-IV phenotyping. However, repeating the experiment presented in Figs. 1A and B (pH range 5 to 7) by using a pH range of 4 to 6 did not result in different apoA-IV isoelectric focusing patterns (results not shown). Therefore, we conclude that apoA-IV phenotyping can be reliably performed using a pH range of 5 to 7. **Fig. 2** shows that immuno-staining of apoA-IV isoelectric focusing patterns can also be performed with apoE immunoblots that have been stored in the dark for more than 1 year.

Consequently, the immunoblots that arose from our earlier population study on apoE phenotype distribution could easily be re-used for screening for the apoA-IV polymorphism.

ApoA-IV isoforms and phenotype and allele frequency distribution

According to the nomenclature introduced by Menzel et al. (12), by far the most frequent isoform is indicated

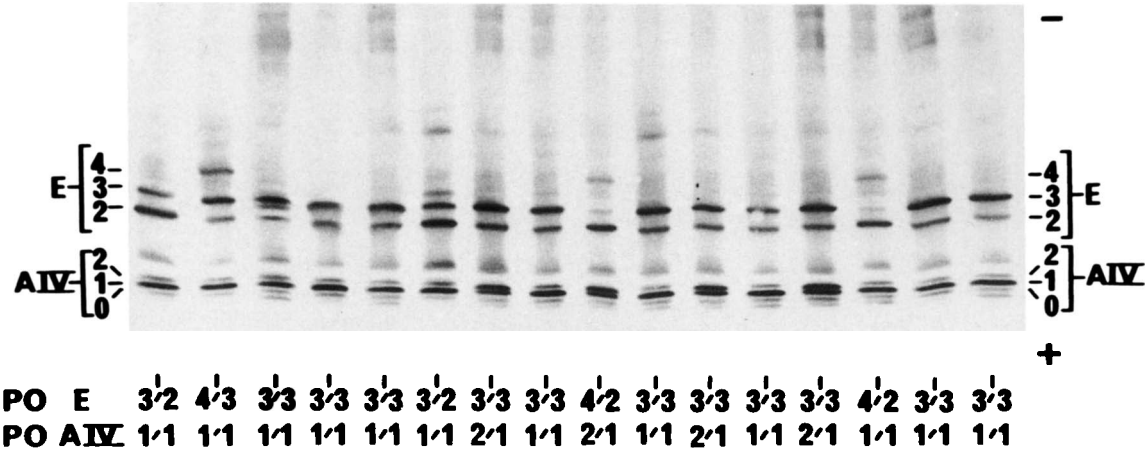

Fig. 2. Immuno-visualization of apoA-IV isoelectric focusing patterns using a preexisting apoE immunoblot. The apoE immunoblot (pH range 5 to 7) had been stored in the dark for more than 1 year.

TABLE 1. ApoA-IV phenotype and relative gene frequency distribution in a population sample of 1,393 randomly selected 35-year-old males

Phenotype	Number[a]		Relative Frequency	
	Observed	Expected	Observed	Expected
A-IV-1/A-IV-1	1,127	1129.8	0.8090	0.8111
A-IV-1/A-IV-2	200	199.0	0.1436	0.1429
A-IV-1/A-IV-3	50	45.03	0.0359	0.0323
A-IV-1/A-IV-0	5	5.40	0.0036	0.0039
A-IV-2/A-IV-2	10	8.77	0.0071	0.0063
A-IV-2/A-IV-3	0	3.97	0	0.0029
A-IV-2/A-IV-0	1	0.48	0.0007	0.0003
A-IV-3/A-IV-3	0	0.45	0	0.0003
A-IV-3/A-IV-0	0	0.11	0	0.0001
A-IV-0/A-IV-0	0	0.01	0	0
Total	1,393		1.0000	

Allele	Relative Gene Frequencies
A-IV*0	0.002
A-IV*1	0.901
A-IV*2	0.079
A-IV*3	0.018

[a]The distribution of the different apoA-IV phenotypes is in Hardy-Weinberg equilibrium (χ^2 = 5.9; df = 9).

as A-IV-1. The less frequent but still common isoform basic to A-IV-1 is designated as A-IV-2. We also found a rare A-IV isoform slightly more acidic than A-IV-1. We designated this isoform as A-IV-0 (see Fig. 1B).

A rare isoform slightly more basic than A-IV-2 has also been found (designated as A-IV-3).

By family studies (15 meioses) we could demonstrate Mendelian inheritance for A-IV-0, A-IV-1, and A-IV-2 (results not shown). For A-IV-3, a family was not available in order to evaluate whether this isoform is inherited in a Mendelian fashion.

The apoA-IV phenotype distribution and the APOA-IV gene frequencies are presented in **Table 1.** The distribution of the different apoA-IV phenotypes in this population sample was not statistically different from the expected Hardy-Weinberg distribution (χ^2 = 5.9; df = 9). From this table, it may be concluded that the A-IV*1 and A-IV*2 alleles are by far the most common APOA-IV alleles with gene frequencies of 0.901 and 0.079, respectively.

Effect of allele substitutions at the APOA-IV gene locus on plasma cholesterol, triglyceride, apoB, and apoE levels

Mean values of plasma cholesterol, triglycerides, apoB, and apoE levels are presented in **Table 2** for each apoA-IV phenotypic group. No statistically significant differences could be found among the different apoA-IV phenotypic groups using both parametric and nonparametric statistical tests (see Materials and Methods).

ApoE phenotype distribution affects plasma cholesterol, apoB and apoE levels (24-27). Since in the present paper we screened a population that had previously been screened for the apoE polymorphism, we were able to calculate the apoE phenotype distribution within the different apoA-IV phenotypic groups (**Table 3**). Chi-square

TABLE 2. Plasma cholesterol, triglyceride, apoB, and apoE concentrations in different apoA-IV phenotypic groups (mean ± SD)

Phenotype	Number	Cholesterol	Triglyceride	ApoB	ApoE
		mmol/l		*mg/dl*	
A-IV-1/A-IV-1	1127	5.40 ± 0.98	1.69 ± 1.07	116 ± 35	5.63 ± 2.42
A-IV-1/A-IV-2	200	5.50 ± 1.10	1.67 ± 1.00	116 ± 39	5.59 ± 2.07
A-IV-1/A-IV-3	50	5.65 ± 1.24	1.61 ± 0.85	115 ± 36	5.63 ± 2.39
A-IV-1/A-IV-0	5	5.01 ± 0.98	1.59 ± 0.71	103 ± 35	8.61 ± 6.21
A-IV-2/A-IV-2	10	5.42 ± 1.31	1.95 ± 0.98	121 ± 40	6.39 ± 1.34
A-IV-2/A-IV-0	1	9.41	1.91	220	8.84
Total population	1393	5.43 ± 1.02	1.68 ± 1.05	116 ± 36	5.64 ± 2.39

TABLE 3. ApoE phenotype distribution within different apoA-IV phenotypic groups

ApoA-IV Phenotype	ApoE Phenotype (Observed/Expected)						Total Number
	4/4	4/3	4/2	3/3	3/2	2/2	
A-IV-1/A-IV-1	23/27.5	301/300	30/26.7	627/620.5	140/144.8	6/7.3	1127
A-IV-2/A-IV-1	10/4.9	51/53.3	3/4.7	104/110	31/25.7	1/1.3	200
A-IV-2/A-IV-2	0/0.2	3/2.7	0/0.2	6/5.5	1/1.3	0/0.1	10
A-IV-3/A-IV-1	1/1.2	14/13.3	0/1.2	27/27.5	7/6.9	1/0.3	50
Others[a]	0/0.2	2/1.6	0/0.1	3/3.3	0/0.8	1/0.0	6
Total number	34	371	33	767	179	9	1393

[a]Because of low numbers, the groups of A-IV-2/A-IV-0 and A-IV-1/A-IV-0 phenotypes are combined into one group.

analysis revealed that there is no linkage dysequilibrium between alleles in the APOA-IV and APOE locus.

Table 4 presents the estimates of the relative contribution of the APOA-IV gene to the total phenotypic variation of the measured lipoprotein parameters, calculated as described by Sing and Davignon (24). The genetic variance contributed only about 0.1% to the total population variability in plasma cholesterol levels. The contribution of the genetic variance associated with the APOA-IV gene locus to the total phenotypic variance in levels of triglyceride, apoB, and apoE is less than 0.1% (0.044, 0.012 and 0.075%, respectively).

Effect of apoA-IV polymorphism on plasma levels of apoA-IV and A-I

To evaluate whether the polymorphism of apoA-IV affects its own plasma level and the level of HDL, we measured the concentrations of apoA-IV and A-I in a number of randomly selected subjects within each apoA-IV phenotypic group. From the results presented in **Table 5** it is obvious that in the A-IV-1/A-IV-3 phenotypic group the level of apoA-IV is significantly lower than in the other groups. A statistically significant effect of the apoA-IV polymorphism on plasma apoA-I levels was not found. Furthermore, a statistically significant correlation between apoA-IV levels on one hand and all the other measured lipid and apolipoprotein levels on the other hand could not be found.

TABLE 4. Relative contribution of the genetic variance associated with the APOA-IV gene locus to the total phenotypic variance

Lipoprotein Parameter	Genetic Variance Associated with the APOA-IV Locus	Total Phenotypic Variance (Square Standard Deviation)
	% of phenotypic variance	*(mg/dl)²*
Cholesterol	0.106	1497
Triglyceride	0.044	9966
ApoB	0.012	1298
ApoE	0.075	5.6

DISCUSSION

Several groups of investigators have reported that human apoA-IV can be separated by isoelectric focusing into two major isoforms (12,14), whereas far less common isoforms of apoA-IV have also been found (12,15). Kamboh and Ferrell (14) have reported that besides genetic variation, intra-individual microheterogeneity of apoA-IV also occurs, possibly due to posttranslational modification such as glycosylation, deamidation, sulfhydration, or proteolytic processing (7). The minor bands seen in our apoA-IV isoelectric focusing patterns (Figs. 1 and 2) also point to the existence of an intraindividual microheterogeneity of apoA-IV.

In this study we present data on the genetic polymorphism of apoA-IV in a Dutch population sample of 1,393 randomly selected 35-year-old male individuals. This population sample had been used earlier for the determination of apoE phenotype and gene distribution (18,26) by employing isoelectric focusing (pH range 5 to 7) of delipidated plasma samples, followed by immunoblotting using anti-apoE antiserum as first antibody (19). Our present results show that the apoE immunoblots that arose from that population study could be easily used for immuno-visualization of apoA-IV isoelectric focusing patterns afterwards, even after storage for more than 1 year in the dark (Fig. 2). This observation enabled us to screen efficiently for apoA-IV polymorphism in a relatively large population sample. From this population study we conclude that at least four distinct apoA-IV isoforms exist. The two common isoforms with relative allele frequencies of 0.901 and 0.079 are indicated as A-IV-1 and A-IV-2, respectively, according to the nomenclature introduced by Menzel et al. (12). Consequently, we designated the rare apoA-IV isoforms acidic to A-IV-1 and basic to apoA-IV-2 as A-IV-0 and A-IV-3, respectively. ApoA-IV-0 is possibly similar to the rare acidic isoform described by Bisgaier et al. (15), whereas apoA-IV-3 might be identical to apoA-IV-Münster described by Menzel et al. (12).

Our results show that the relative frequencies of the two common A-IV*1 and A-IV*2 alleles (Table 1) are very similar to the allele frequencies reported by others (12, 14,

TABLE 5. Plasma apoA-IV and A-I concentrations in different apoA-IV phenotypic groups (mean ± SD)

Phenotype	Number Measured	ApoA-IV	ApoA-I
		mg/dl	
A-IV-1/A-IV-1	100	14.9 ± 4.6	133.2 ± 17.6
A-IV-1/A-IV-2	100	14.5 ± 4.4	138.3 ± 20.1
A-IV-1/A-IV-3	49	4.8 ± 2.8[a]	135.9 ± 20.7
A-IV-1/A-IV-0	4	14.8 ± 3.7	133.5 ± 17.2
A-IV-2/A-IV-2	10	12.8 ± 4.0	137.4 ± 11.5
Total	263	12.8 ± 5.7	135.8 ± 19.1

[a]Different from other groups as evaluated by Student's *t*-test for unpaired samples.

28). This close similarity, together with the observation that the apoA-IV phenotype distribution in our population sample did not differ from the expected Hardy-Weinberg distribution (x^2 = 5.9; df = 9), supports our claim that apoA-IV polymorphism can be determined easily and reliably following immunoblotting for apoE phenotyping.

It has been suggested that apoA-IV is involved in chylomicron synthesis and/or secretion (8,9). However, a possible influence of apoA-IV polymorphism on plasma lipid and lipoprotein parameters has not yet been reported.

Our results show that no statistically significant difference could be found between the different apoA-IV phenotypic groups in either plasma levels of cholesterol, triglyceride, apoB or apoE (Tables 2 and 3). Using the formula described by Sing and Davignon (24), we also found that for all lipoprotein parameters considered in this paper, 0.1% or less of the inter-individual variability (phenotypic variance) is explained by allele substitutions at the APOA-IV gene locus (Table 4). This finding is in sharp contrast with the previously observed strong association of these lipoprotein parameters with apoE polymorphism (24–27). Besides an effect in chylomicron synthesis and/or secretion, apoA-IV also seems to be involved in processes facilitating reverse cholesterol transport as it activates lecithin:cholesterol acyltransferase (29,30) and it determines the binding and uptake of rat HDL by rat hepatocytes (31).

Our results did not show any influence of the apoA-IV polymorphism on plasma levels of apoA-I, the major protein component of HDL and lymph chylomicrons, nor could we find any correlation between apoA-I and apoA-IV levels within the different apoA-IV phenotypic groups. This is in agreement with the results reported by Elshourbagy et al. (32) and Steinmetz et al. (33). Both groups showed that the synthesis and levels of both apolipoproteins are independently regulated in rat (32) and man (33).

Steinmetz et al. (33) found that apoA-IV levels in human neonates dramatically increased to adult levels during the first week of life (increment from about 7 to 17 mg/dl). Our results show that subjects bearing the AIV*3 allele exhibit significantly lower levels of plasma apoA-IV than subjects without the AIV*3 allele (5 mg/dl versus about 14 mg/dl). Whether the lower level of apoA-IV in these subjects is due to an influence of the apoA-IV polymorphism on the induction of apoA-IV postpartum, is currently under investigation.

The authors gratefully acknowledge Jo Bury for providing us with his expertise and knowledge concerning large scale apolipoprotein B and E measurements. We thank Mrs. Clara Horsting-Been and Miss Marisa Horsting for preparing the manuscript. These investigations were supported by the Division for Health Research TNO (project no. 900-504-059).

Manuscript received 30 January 1988 and in revised form 23 June 1988.

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