Biochemical and Genetic Association of Plasma Apolipoprotein A-II Levels With Familial Combined Hyperlipidemia

Hooman Allayee, Lawrence W. Castellani, Rita M. Cantor, Tjerk W.A. de Bruin, Aldons J. Lusis

Abstract—Apolipoprotein A-II (apoA-II) is a major protein on high-density lipoprotein (HDL) particles, and in mice, its levels are associated with triglyceride and glucose metabolism. In particular, transgenic mice overexpressing apoA-II exhibit hypertriglyceridemia, increased body fat, and insulin resistance, whereas apoA-II–null mice have decreased triglycerides and increased insulin sensitivity. Given the phenotypic overlap between familial combined hyperlipidemia (FCH) and apoA-II transgenic mice, we investigated the relationship of apoA-II to this disorder. Despite having lower HDL-cholesterol (HDL-C), FCH subjects had higher apoA-II levels compared with unaffected relatives (P<0.00016). Triglyceride and HDL-C levels were significant predictors of apoA-II, demonstrating that apoA-II variation is associated with several FCH-related traits. After adjustment for multiple covariates, there was evidence for the heritability of apoA-II levels (h²=0.15; P<0.02) in this sample. A genome scan for apoA-II levels identified significant evidence (LOD=3.1) for linkage to a locus on chromosome 1q41, coincident with a suggestive linkage for triglycerides (LOD score=1.4). Thus, this locus may have pleiotropic effects on apoA-II and FCH traits. Our results demonstrate that apoA-II is biochemically and genetically associated with FCH and may serve as a useful marker for understanding the mechanism by which FCH develops. (Circ Res. 2003;92:695–701.)

Key Words: apolipoprotein A-II ■ genetics ■ metabolic syndrome ■ triglycerides ■ insulin resistance

A polipoprotein A-II (apoA-II) is the second most abundant protein on HDL particles but has not been reported to have a clear function. Its presence on HDL either decreases or has no influence on cholesterol efflux from cells,1–3 and rare individuals lacking apoA-II appear to have relatively normal plasma lipid levels.4 We previously reported that overexpression of apoA-II in mice increased atherosclerosis despite a marked elevation in HDL-C.5 Subsequently, we also demonstrated that apoA-II transgenic mice exhibit 10-fold higher plasma triglyceride levels, elevated free fatty acids, altered body fat homeostasis, and dramatic insulin resistance compared with control animals.6–8 ApoA-II–null mice, on the other hand, exhibit opposite phenotypes, including increased insulin sensitivity and a decreased atherogenic lipid profile.7 Taken together, these observations in mice suggest that apoA-II metabolism can contribute to various metabolic disturbances associated with insulin resistance syndromes. Moreover, genome scans for type 2 diabetes in several populations have observed linkage to the apoA-II gene locus8–10 and polymorphisms of the apoA-II gene have been associated with free fatty acid and triglyceride levels.11,12

ApoA-II transgenic mice also have characteristics that resemble the common human disorder familial combined hyperlipidemia (FCH). Although FCH patients typically present with hypertriglyceridemia and/or hypercholesterolemia, such individuals frequently also exhibit insulin resistance, altered fatty acid metabolism, central adiposity, and other metabolic disturbances that, together, presumably account for the high rate of premature coronary artery disease (CAD) in these patients.13,14 In fact, overexpression of human apoA-II in apoE-deficient mice has been reported to serve as a phenotypic model for FCH, lending support for the notion that apoA-II could play a role in this disorder.15

In the present study, we characterized 18 Dutch FCH families16 for plasma apoA-II levels and performed a genome screen to identify chromosomal regions harboring putative genes that contribute to apoA-II variation in this altered metabolic state. The results demonstrate that apoA-II levels are higher in FCH subjects and are positively associated with the levels of other plasma lipids. We also report the identification of several loci that segregate with apoA-II levels in these Dutch families.

Materials and Methods

Ascertainment of FCH Families

Eighteen extended FCH families of Dutch Caucasian descent were ascertained through probands recruited from the Lipid Clinic of the
Utrecht Academic University Hospital, as previously described.\textsuperscript{16–18} The probands met the following criteria: (1) a primary combined hyperlipidemia with varying phenotypic expression, including a fasting plasma cholesterol $>6.5$ mmol/L, or $>90$th percentile for age, defined according to tables from the Lipid Research Clinics, and fasting plasma triglycerides $>2.3$ mmol/L; (2) at least one first degree relative with a different hyperlipidemic phenotype; and (3) a positive family history of premature CAD defined as a myocardial infarction or cardiovascular disease before 60 years of age. Although plasma apolipoprotein B (apoB) levels are also frequently elevated in FCH individuals and sometimes used as another diagnostic feature, this latter criterion was not applied in the ascertainment of these families. Exclusion criteria for the probands included diabetes, obesity (BMI $>30$), tendon xanthomas, or type III hyperlipidemia (apoE2/E2). Over 95% of the relatives and spouses of the probands were also collected without regard to FCH affection status. Relatives were assigned the FCH phenotype when they met the following criteria: fasting plasma cholesterol $>6.5$ mmol/L, and/or fasting plasma triglycerides $>2.3$ mmol/L. Using these criteria, there were 158 affected individuals and 212 unaffected relatives in these pedigrees. Table 1 represents an environment-, nutrition-, and age-matched control group for the probands and their hyperlipidemic relatives. All subjects gave informed consent, and the study protocol was approved by the Human Investigation Review Committees of Utrecht University Hospital, the Netherlands, and the University of California, Los Angeles.

**Laboratory Analytical Methods**

Venous blood was drawn after an overnight fast of 12 to 14 hours, and plasma was prepared by immediate centrifugation. Lipids, apolipoproteins, and measures of insulin resistance/glucose metabolism were quantified by methods as described elsewhere.\textsuperscript{17,18} Plasma apoA-II levels were determined in duplicate by an endpoint nephelometric assay using a Molecular Devices Spectramax Plus microplate reader. Three control samples with known apoA-II concentrations (Quantimetrix Corporation and International Immunology) were analyzed in each assay to ensure accuracy. ApoA-II antibodies, calibrator standards, and buffers were obtained from Wako Chemicals USA. Performance characteristics for this assay are as follows: average within-run CV $4.08$; average run-to-run CV, $4.31$. Probands and hyperlipidemic relatives who used lipid-lowering drugs were studied after their lipid lowering treatment was withheld for 3 weeks. Only these lipid values and other quantitative measurements were used in this study. Alcohol use was not controlled for, beyond the subjects' abstention from alcohol consumption for at least 48 hours before having blood drawn.

**Genotyping**

For the genome scan, 399 microsatellite markers, spaced on average 10 cM, were genotyped in 240 individuals from the 18 FCH families using fluorescent-based methods, as described elsewhere.\textsuperscript{20,21} We previously conducted a genome scan for the discrete FCH trait in these same families, and in the present study, the same genotyping data were used to conduct a genome scan for plasma apoA-II levels. The markers used in construction of the linkage map comprised the Weber 6a screening set, and the heterozygosity indices of the markers averaged 0.82 in the families analyzed.

**Genetic Statistical Analyses**

To assess the relationship between apoA-II levels and other metabolic traits related to FCH, we conducted regression analyses using trait values from all phenotyped members of the 18 pedigrees. To account for the nonindependence of family members, we used a generalized estimating equation approach, as programmed in the GENMOD procedure of the Statistical Analysis System (SAS/STAT Software: Changes and Enhancements through release 6.12, SAS Institute Inc, 1997). GENMOD has the advantage of including a parameter in the analysis to model the correlation among family members.\textsuperscript{22} Sex, age, triglycerides, total cholesterol, LDL-C, HDL-C, apoB, FFA, apoA-I, insulin, glucose, and BMI were all entered into this regression analysis as predictors of apoA-II. Plasma triglyceride and insulin levels were log transformed before these and all subsequent analyses due to nonnormality. Potential predictors of apoA-II levels were interpreted as significant if the regression coefficient had a value $P<0.05$. To assess differences in each FCH characteristic between affected and unaffected relatives and to estimate the contribution of genetic factors (heritability) to plasma apoA-II levels, a quantitative likelihood polygenic analysis was performed. SOLAR (v. 1.7.3) was used to incorporate the familial relationships in these large and complex pedigrees,\textsuperscript{23} and a variance components analysis of family data was performed, which decomposes the total variance of the phenotype into components due to genetic effects, covariates, and random environmental effects. “Affected” status was included as a binary covariate, and differences in mean lipid, apolipoprotein, and insulin resistance traits were assessed between the FCH affected and unaffected relatives. Trait differences between hyperlipidemics and normolipidemics were considered significant if the value for this analysis was $P<0.05$. The contribution of genetic factors to apoA-II variation was estimated by calculating the heritability ($h^2$). Significance of $h^2$ values was assessed by a likelihood ratio test, in which the likelihood of the model with an additive genetic variance component and covariates was compared with the likelihood model in which the additive genetic variance component was constrained to be 0. However, $h^2$ may be overestimated because only known covariates are entered into the model.

The original genome scan study was designed so that individuals comprising the largest and most informative sibships from the 18 families were genotyped.\textsuperscript{21} Given the nature of this genotyping data and the presumed complex inheritance of apoA-II levels, nonparametric, sibpair allele sharing methods were used for the present linkage analyses of the apoA-II data. The MAPMAKER/SIBS program was used to assess multipoint linkage between marker loci and quantitative traits.\textsuperscript{24} This program uses information from multiple, linked markers to estimate allele sharing at and between markers. The test statistic for a correlation between allele sharing and squared trait differences among sibpairs ($n=322$) is reported as a LOD score.\textsuperscript{25} In addition, this multipoint analysis mitigates, in part, the potential effect of markers with low heterozygosity indices, because the informativeness of a locus is also calculated using linked markers.

**Electronic Database Information**

Accession numbers and URLs for data in this article are as follows: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for FCH, MIM 144250); and Marshfield Center for Human Genetics, http://www.marshmed.org/genetics (for genome screen marker information).

**Results**

The clinical characteristics of the 18 FCH families are described in Table 1. As expected, the FCH probands and hyperlipidemic relatives exhibited higher plasma total cholesterol, triglyceride, and apoB levels, while having lower HDL-C compared with the unaffected family members ($P<0.001$). However, as the FCH phenotype is typically not manifested until the second decade of life, it cannot be excluded that some of the normolipidemic relatives, who are younger compared with the affected relatives, have yet to fully express the disease trait. The affected individuals also had higher plasma levels of insulin, glucose, and free fatty acids, supporting the notion that FCH has overlapping features with type 2 diabetes and the metabolic syndrome. These clinical differences remained significant after adjustment for age and sex (data not shown). Interestingly, apoA-I levels were not significantly different between the hyperlipidemias and normolipidemics. In addition, there was no significant
To assess the role that apoA-II may play in FCH, we measured apoA-II levels in 472 members of the 18 families for whom plasma was available. The distribution of plasma apoA-II levels in the hyperlipidemic (Figure 1A), normolipidemic (Figure 1B), and spouse controls (Figure 1C) ranged from 11 to 65 mg/dL and did not significantly deviate from normality in any of the groups. Using variance components analysis, apoA-II levels were examined by affected status and found to be slightly, but significantly, higher in the hyperlipidemic (Figure 1A), normolipidemic (Figure 1B), and spouse controls (Figure 1C) groups (Table 1). This observation is consistent with what would be expected based on the hypertriglyceridemia that apoA-II transgenic mice exhibit. The difference between the affected and unaffected individuals was even more pronounced when the apoA-II/HDL-C ratio was examined (34.8 versus 28.7; \( P < 0.0001 \)) and indicates that the HDL in FCH is enriched for apoA-II.

To assess whether apoA-II levels were quantitatively associated with FCH characteristics, we performed stepwise regression analyses with various lipids, including total cholesterol, triglycerides, HDL-C, as well as other metabolic parameters, using a generalized estimating equation. After accounting for the nonindependence among family members, apoA-II levels, as predicted by FCH traits, showed a significant linear relationship with age (\( P < 0.0006 \)), triglyceride (\( P < 0.0001 \)), HDL-C (\( P < 0.0001 \)), apoA-I (\( P < 0.0001 \)), and apoB levels (\( P < 0.004 \)) (Table 2). The overall scaled covariance statistic of 1.03 from these analyses signifies that this model fit the data well and indicates that apoA-II is perturbed along with the other important lipid traits that are part of the FCH phenotype. The insulin resistance features of FCH, including elevated free fatty acids, insulin, glucose, and BMI, did not significantly predict apoA-II levels. Thus, although apoA-II is associated with the traits used for ascertaining FCH, it does not appear to be an integral feature of the insulin resistance related traits in this population. Lastly, we performed all the regression analyses with the probands excluded to avoid the possibility that these hyperlipidemic individuals were skewing the data. However, the results of these analyses did not significantly differ from those that included the probands with the exception of the association between apoA-II and apoB levels, which became nonsignificant. This may be due to the relationship between apoB and FCH because apoB levels are significantly higher in the probands than unaffected relatives. Thus, when the probands (and elevated apoB levels) are removed from the analysis, it appears that the association between apoA-II and apoB is lost.

The observed association between apoA-II and FCH prompted us to test whether there was a genetic contribution to apoA-II variation in this Dutch FCH cohort. We first determined the heritability (h\(^2\)) of apoA-II levels with all phenotyped individuals (\( n = 472 \)) using variance components analysis. Unadjusted h\(^2\) of apoA-II levels in the 18 families was estimated to be 0.35 (\( P < 0.00002 \)). Including covariates in the model decreased the h\(^2\) estimate to 0.15 (\( P < 0.02 \)), with only age, sex, triglycerides, HDL-C, and apoA-I levels having significant effects on apoA-II. These results are in agreement with the regression analyses described above, and demonstrate that, even after adjustment for covariates, there is still significant evidence for the contribution of genetic factors to apoA-II variation.

Based on the finding that genetic factors are contributing to apoA-II levels in these families, we conducted a genome scan for apoA-II levels on a subset of individuals from the 18 families (\( n = 240 \)) for whom genotyping was previously performed.\(^{21}\) Assuming that the genetic factors contributing to apoA-II variation are most likely complex, the data were analyzed using quantitative, nonparametric sibpair allele sharing linkage methods. We identified four genomic regions that demonstrated evidence of linkage with apoA-II levels but did not observe evidence of linkage to the apoA-II structural gene itself, a finding that has been reported in other studies.\(^{11,26}\) The most dramatic result from our genome scan was

<table>
<thead>
<tr>
<th>Trait</th>
<th>Hyperlipidemic Individuals* (n=176)</th>
<th>Normolipidemic Individuals (n=212)</th>
<th>Spouse Controls (n=173)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>49±16‡</td>
<td>35±16</td>
<td>47±15</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>2.7±1.6‡</td>
<td>1.3±0.42</td>
<td>1.6±1.1</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>7.5±2.5‡</td>
<td>4.9±0.83</td>
<td>5.7±1.1</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.14±0.32‡</td>
<td>1.24±0.29</td>
<td>1.26±0.34</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>132±28‡</td>
<td>83±21</td>
<td>102±29</td>
</tr>
<tr>
<td>ApoA-II, mg/dL</td>
<td>38±7†</td>
<td>35±7</td>
<td>36±7</td>
</tr>
<tr>
<td>ApoA-I, mg/dL</td>
<td>134±26</td>
<td>134±24</td>
<td>139±23</td>
</tr>
<tr>
<td>Free fatty acids, mmol/L</td>
<td>0.56±0.24§</td>
<td>0.51±0.23</td>
<td>0.52±0.25</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.1±1.1†</td>
<td>4.5±0.9</td>
<td>4.8±0.8</td>
</tr>
<tr>
<td>Insulin, pmol/L</td>
<td>65.7±58.6†</td>
<td>46.8±33.4</td>
<td>52.6±61.2</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD.

*Includes the 18 probands and 158 hyperlipidemic individuals.
†ApoA-II levels were only measured in 154 hyperlipidemic individuals, 181 normolipidemics, and 137 spouses.
‡P<0.001 and §P<0.01 between hyperlipidemic and normolipidemic groups.
obtained on the long arm of chromosome 1, where a significant LOD score of 3.1 was observed between markers D1S1678 and D1S2141 (Figure 2). Interestingly, evidence for linkage of triglycerides to this locus was also observed with a LOD score of 1.4. The other three loci exhibited suggestive evidence for linkage (LOD scores >1.0) and were located on chromosomes 3, 5, and 17 (Figure 3). In contrast to the chromosome 1q41 locus, linkage of apoA-II-associated traits, such as triglycerides, HDL-C, and apoA-I, was not observed to any of these three loci. Taken together, these results support the notion that genetic factors contribute to apoA-II variation in FCH families and suggest that a subset of these loci provide a genetic basis for the association between apoA-II and certain FCH traits.

Discussion

FCH is a complex genetic disorder whose underlying defect at the biochemical or genetic level has remained elusive despite 30 years of investigation. FCH patients typically manifest with elevated triglyceride and cholesterol levels but very often also exhibit insulin resistance and altered fatty acid metabolism.13 Given the clustering of such abnormalities, FCH shares features with type 2 diabetes and the insulin resistance or metabolic syndrome. Thus, identification of biochemical or genetic markers involved in the metabolic disturbances associated with such diseases may help to unravel their complexity. This approach can be complemented using animal models. Because apoA-II transgenic mice and apoA-II–null mice exhibit phenotypes that are relevant to human metabolic disorders,6,7 we examined plasma apoA-II levels in FCH families to determine whether this HDL-associated protein could play a role in the etiology of this disease. The results suggest that apoA-II variation is associated with certain, but not all, features of FCH and provide evidence for a genetic component to this association.

Figure 1. Frequency distribution of plasma apoA-II levels in the hyperlipidemic (A), normolipidemic (B), and spouse control (C) groups from the 18 FCH families.
Using regression analysis, we observed that apoA-II variation within these families is predicted by core FCH traits, such as triglycerides and total cholesterol, whereas fasting insulin, glucose, and BMI are not good predictors. Therefore, although apoA-II is associated with the primary dyslipidemia in FCH, its association with the insulin resistance characteristics may be obscured by the complex etiology and heterogeneity of such metabolic disturbances in this disorder. This notion is consistent with our recent studies demonstrating that insulin resistance in FCH is metabolically different from that in type 2 diabetes.27 Moreover, these regression analysis results differ from what is observed in apoA-II transgenics because these mice exhibit both hypertriglyceridemia and insulin resistance and it is known that apoA-II is the causal basis for these abnormalities.6 However, we are not able to infer whether the same causal relationship exists in this population.

It is not surprising that apoA-II and HDL-C levels are strongly correlated given that apoA-II is an abundant HDL protein that has previously been demonstrated to influence HDL-C levels.5 It is also known that FCH individuals tend to have lower HDL-C, although this has not traditionally been used as a diagnostic factor.13 We observed that apoA-II levels are higher in the family members who have been classified as FCH compared with unaffected family members. Thus, the probands and affected family members actually have higher apoA-II levels despite having lower HDL-C. Because apoA-II–enriched HDL has been shown to be more proinflammatory compared with normal HDL,2,28 the altered apoA-II to HDL-C ratio in the hyperlipidemic group can be considered more atherogenic compared with the unaffected relatives (and spouse controls) and may serve as another marker for the altered lipid metabolism in FCH.

A potential mechanism for the correlation between triglycerides and apoA-II levels may be the result of the reaction catalyzed by cholesteryl ester transfer protein (CETP). For example, hypertriglyceridemia favors the transfer of triglycerides back to HDL via CETP, generating triglyceride-rich HDL, which are better substrates for hepatic lipase. This would in turn lead to smaller HDL3 particles and create a better vehicle for apoA-II, because it is known that most of the plasma apoA-II is found on smaller HDL. Thus, elevated triglycerides, such as that in FCH, could potentially lead to higher steady state concentrations of plasma apoA-II levels and account, in part, for the observed differences between the affected and unaffected individuals.

In the present study, we identified an apoA-II locus on chromosome 1q41 in insulin resistant, FCH families. Notably, this same region was also linked to apoA-II levels in insulin-resistant Hispanic families ascertained through hypertensive probands.29 The peak region of linkage in both studies was centered around marker D1S2141, both loci were detected using model-free analyses, and both yielded LOD scores exceeding 3.0, the conventional criterion for statistical

<table>
<thead>
<tr>
<th>Trait</th>
<th></th>
<th>(p)</th>
<th>(p^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.0006</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>ApoA-I</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>ApoB</td>
<td>0.004</td>
<td>NS</td>
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</tr>
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<td>BMI</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Free fatty acids</td>
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<tr>
<td>Glucose</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>NS</td>
<td>NS</td>
<td></td>
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</table>

*Excludes probands from the analysis. NS indicates not significant.

Figure 2: A locus on chromosome 1q41 for plasma apoA-II and triglycerides levels. Multipoint linkage was calculated by the MAPMAKER/SIBS program and the order of the markers along the chromosomes is taken from maps provided by the Marshfield Medical Research Foundation.
The suggestive linkage of triglycerides to this locus in the Dutch population also raises the possibility that the putative gene(s) may have pleiotropic contributions to lipid and apoA-II metabolism. Furthermore, this is consistent with the regression analyses, which demonstrated that triglycerides, but not insulin-related traits, were a significant predictor of apoA-II in this population. Because the apoA-II locus on 1q41 was not observed in randomly ascertained families, other insulin resistance predisposing genes may be necessary in order to detect its effect. In this regard, it would be encouraging for this locus to be replicated in other insulin resistant (and normal) populations as well.

FCH and the diseases with which it overlaps are genetically complex and will most likely be due to multiple genes as well as important contributions from environmental factors. Several chromosomal loci and a few genes with modest effect have been associated with FCH or its component traits, but the major genetic factors contributing to FCH have yet to be determined. Thus, novel biochemical abnormalities and/or genetic factors need to be identified in order to elucidate the mechanism by which FCH develops and how this increases the risk of premature CAD. One such factor may involve apoA-II, given the number of studies, particularly in mice, demonstrating that elevated levels of this protein play a detrimental role in lipid homeostasis and atherosclerosis susceptibility. In this study, we identified several chromosomal loci associated with plasma apoA-II levels in FCH families. These do not overlap with loci that have been reported for FCH itself, suggesting that apoA-II, although associated with FCH, is not one of the primary genetic causes. This would be consistent with biochemical studies demonstrating that FCH patients have a 2-fold increase in VLDL production, which is unlikely to be due to increased apoA-II. Nevertheless, identification of genes that control apoA-II levels could help break apart the genetic factors leading to FCH (and other disorders), and may lead to an understanding of the mechanism by which this apolipoprotein leads to altered metabolic states.

Acknowledgments
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References


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