Alanine Aminotransferase as an Independent Predictor of Incident Nonalcoholic Fatty Liver Disease

To the Editor:
We read with interest the recent article by Chang et al. (1) reporting that higher serum alanine aminotransferase (ALT) concentrations, within the reference interval, independently predicted the incidence of nonalcoholic fatty liver disease (NAFLD) during a mean follow-up of 2.5 years in a large cohort of apparently healthy Korean men.

Various prospective studies have previously shown that increased ALT concentrations, even within the reference interval, also predict the future development of type 2 diabetes (2) and cardiovascular events (3) independently of other known risk factors. In all of these studies, however, increased ALT concentrations have been used as a surrogate marker of NAFLD. Indeed, increased liver enzymes are usually thought to be a consequence (and not a cause) of liver injury in NAFLD and can possibly be used as predictors of NAFLD progression (2).

We think the association of the 2 conditions, increased ALT concentrations, and incident NAFLD, does not necessarily prove causation. The greater incidence of NAFLD—as diagnosed by ultrasound—among those with slightly increased ALT concentrations at baseline is most likely attributable to an underlying common mechanism, i.e., more severe insulin resistance in those with higher than in those with lower ALT concentrations. That the association between increasing serum ALT concentrations and incident NAFLD remained statistically significant even after adjustment for the homeostasis model assessment (HOMA)-estimated insulin resistance may be due simply to the fact that the HOMA score is not a good proxy measure of insulin resistance. Thus, we wonder how different the results would have been if the euglycemic clamp technique or methods that are more accurate had been used to measure insulin resistance.

Interpretation of the results of the Chang et al. study are limited by another major caveat (1), also recognized by the authors, that the diagnosis or exclusion of NAFLD, both at baseline and follow-up, was based on liver enzymes and ultrasound imaging, but was not confirmed by liver biopsy. Indeed, it is known that liver enzymes may be within the reference interval in up to 70% of patients with diagnosed NAFLD and that the full histopathological spectrum of NAFLD may be present in patients with normal liver enzymes, which therefore cannot be reliably used to exclude the presence of NAFLD (2). Moreover, although liver ultrasonography is widely used for diagnosing NAFLD, this imaging method has good sensitivity and specificity only for detection of moderate and severe hepatic steatosis, but its sensitivity is reduced when hepatic fat infiltration on liver biopsy is <33% (4). Only liver biopsy can be used for diagnosing NAFLD and accurately determining the histological severity and prognosis of liver damage (2). Thus, although this limitation, if present, would probably tend to reduce (at least partly) the strength of the association between ALT concentrations and incident NAFLD shown by Chang et al. (1), we think some nondifferential misclassification of NAFLD on the basis of liver enzymes and ultrasonography is likely. That is, some of the study participants may not have NAFLD despite ultrasound detection of fatty liver and some may have underlying NAFLD with normal liver enzymes and negative ultrason sound findings. The latter situation may have been partly confirmed during the short follow-up study (<3 years) by the unexpectedly high incidence of NAFLD detected in the 5237 healthy participants with a mean age of 36 years (984 incident NAFLD cases, i.e., ~75 new cases per 1000 person-years). In contrast, the incidence of high aminotransferase concentrations, as surrogate markers of NAFLD, was recently reported to be ~30 new incident cases per 1000 person-years in a cohort of Japanese healthy individuals (age 35 years) who were free of NAFLD at baseline (5).

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References

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Reply to Alanine Aminotransferase as an Independent Predictor of Incident Nonalcoholic Fatty Liver Disease

To the Editor:
First, the relationship between alanine aminotransferase (ALT) and
nonalcoholic fatty liver disease (NAFLD) has not yet been clearly established, although increased liver enzymes are usually used as a surrogate marker of NAFLD. Tagher et al. have also noted that liver enzymes may be within the reference interval in up to 70% of patients diagnosed with NAFLD and that the full histological spectrum of NAFLD may be present in patients with normal liver enzymes. Our observations were therefore novel and have potential importance in understanding the relationship between ALT and NAFLD. Furthermore, several issues need to be clarified about ALT, including the isoforms of ALT and the role of different ALT isoforms in humans (1).

Second, regarding the adjustment of confounders, such as insulin resistance, we did not perform this study using the euglycemic clamp and cannot comment on Tagher’s concerns about the interrelation between ALT, NAFLD, and insulin resistance as assessed by the euglycemic clamp technique. Indeed, performance of studies with large samples are not feasible with the glucose clamp methods. Fortunately, other investigators have reported that the homeostasis model assessment of insulin resistance (HOMA-IR) is strongly related to clamp-measured insulin resistance (2). Furthermore, a recent study using sophisticated techniques such as MRI and the oral glucose tolerance test has shown that only half of the patients with MRI-assessed fatty liver disease had increased ALT, whereas even modest ALT increases within the reference interval were associated with deterioration in glucose and lipid metabolism as well as insulin resistance (3). As we noted in our article, even in participants without any features of the metabolic syndrome, any increase of serum ALT, even that within the reference interval, continued to predict the incidence of NAFLD (4). To date, although the relationship between ALT and NAFLD remains to be elucidated, our observation supports the idea that ALT is not only a consequence but also a predictor of NAFLD. We agree with Tagher and colleagues that insulin resistance as assessed by sophisticated techniques such as the euglycemic clamp method could be helpful in understanding the relationship between ALT and NAFLD through a pathway that is distinct from systemic insulin resistance.

Third, regarding the determination of NAFLD, Tagher et al. raised a concern regarding the ultrasound (US) measurements used in our study, the sensitivity of early fatty infiltration (<33%), and the limited ability, as we also discussed in our article, to differentiate simple steatosis from inflammation or fibrosis (4). Whether the degree of misclassification of fatty liver diagnoses based on US varies according to the ALT concentration is presently unknown. As Tagher et al. note, nondifferential misclassification, if present, would tend to reduce the strength of the associations between ALT and NAFLD.

Finally, Tagher and colleagues found differences in the incidence of NAFLD in our study and a previously reported study by Suzuki et al. (5). These differences may have been in part attributable to differences in both the study populations and the outcome measures. In the study by Suzuki et al. (5), the study participants were 20–59 years old, were 26.8% female, and the incidence of increased transaminasemia was highest among males 20–39 years old (5). Our study population, however, was composed of only males, and the majority (70.8%) were 30–39 years old. Outcome measurements in the Suzuki et al. (5) study were based on increased ALT whereas ours were based on US. For exploratory purposes, we performed ancillary analyses to estimate the incidence of increased ALT as an outcome measure.

| Table 1. Adjusted hazard ratios of incidence of nonalcoholic fatty liver disease in relation to ALT concentrations within the reference interval after excluding the participants who developed NAFLD within the first 1 or 2 years. |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Analyses restricted to the participants after excluding the participants who developed NAFLD within the first 1 year (n = 4826) | Analyses restricted to the participants after excluding the participants who developed NAFLD within the first 2 year (n = 4502) | Analyses restricted to the participants after excluding the participants who developed NAFLD within the first 1 year (n = 4826) | Analyses restricted to the participants after excluding the participants who developed NAFLD within the first 2 year (n = 4502) |
| Cases | 64 | 86 | 115 | 135 | 173 | 20.0 | 2588.1 | 2552.1 | 2326.5 | 2313.3 |
| Person-years | 3200.8 | 2588.1 | 2552.1 | 2326.5 | 2313.3 | 20.0 | 2588.1 | 2552.1 | 2326.5 | 2313.3 |
| ID, (per 1000 person-years) | 20.0 | 33.2 | 45.1 | 58.0 | 74.8 | 20.0 | 33.2 | 45.1 | 58.0 | 74.8 |
| aHR, (95% CI) | 1.00 | 1.65 (1.19–2.28) | 2.25 (1.66–3.06) | 2.90 (2.16–3.91) | 3.84 (2.88–5.12) | 1.00 | 1.34 (0.97–1.88) | 1.55 (1.13–2.14) | 1.95 (1.43–2.65) | 2.09 (1.54–2.84) |
| Model 1 | 1.00 | 1.34 (0.97–1.88) | 1.55 (1.13–2.14) | 1.95 (1.43–2.65) | 2.09 (1.54–2.84) | 1.00 | 1.65 (1.19–2.28) | 2.25 (1.66–3.06) | 2.90 (2.16–3.91) | 3.84 (2.88–5.12) |
| Model 2 | 0.01 | 0.001 | 0.001 | 0.001 |
| Analyses restricted to the participants after excluding the participants who developed NAFLD within the first 1 year (n = 4826) | Analyses restricted to the participants after excluding the participants who developed NAFLD within the first 2 year (n = 4502) |
| Cases | 36 | 40 | 58 | 57 | 58 | 36 | 40 | 58 | 57 | 58 |
| Person-years | 3165.2 | 2530.0 | 2482.9 | 2231.9 | 2174.7 | 3165.2 | 2530.0 | 2482.9 | 2231.9 | 2174.7 |
| ID, (per 1000 person-years) | 11.4 | 15.8 | 23.6 | 25.5 | 26.7 | 11.4 | 15.8 | 23.6 | 25.5 | 26.7 |
| aHR, (95% CI) | 1.00 | 1.35 (0.86–2.12) | 2.05 (1.35–3.11) | 2.21 (1.45–3.35) | 2.44 (1.61–3.70) | 1.00 | 1.26 (0.79–2.02) | 1.68 (1.09–2.60) | 1.74 (1.12–2.69) | 1.65 (1.06–2.57) |
| Model 1 | 1.00 | 1.35 (0.86–2.12) | 2.05 (1.35–3.11) | 2.21 (1.45–3.35) | 2.44 (1.61–3.70) | 0.01 | 0.001 |
| Model 2 | 1.00 | 1.26 (0.79–2.02) | 1.68 (1.09–2.60) | 1.74 (1.12–2.69) | 1.65 (1.06–2.57) |

*Model 1: adjustment for age; Model 2: model 1 plus adjustment for weight change, fasting serum glucose, log, triglyceride, HDL-cholesterol, body mass index, systolic blood pressure, smoking, exercise, alcohol intake, HOMA-IR, C-reactive protein, and incident diabetes. ID, Incidence density; aHR, adjusted hazard ratios.*
Whereas 984 incident cases of US-based NAFLD developed during 13,822.4 person-years of follow-up [incidence density (ID), 74.1 per 1000 person-years; 95% CI, 69.5–78.7], 700 incident cases of increased ALT, defined as serum ALT ≥35 U/L, developed during 13,822.4 person-years of follow-up (ID, 50.6 per 1000 person-years; 95% CI, 46.9–54.4). To minimize the effect of existing fatty infiltration on the baseline ALT, additional analyses were performed by excluding participants with incident NAFLD that occurred during the first 1 or 2 years of follow-up (Table 1). Although the magnitude of the association between ALT and NAFLD was slightly reduced, this analysis did not qualitatively change any of the observed associations (Table 1). Future research will shed light on the question of what underlies the relationship between ALT and an increased risk of NAFLD.

We appreciate the opportunity to clarify our findings. The letter by Tagher and colleagues raises several important issues in the interpretation of our findings.

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References


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Whole Genome Amplification and Genetic Analysis after Extraction of Proteins from Dried Blood Spots

To the Editor:

Programs to screen newborns for congenital disorders are based on analysis of dried blood spot samples (DBSS), which have proven to be robust and convenient for collection, transport, and storage. Because blood samples are collected with no selection, and coverage of the population is essentially universal, residual DBSS combined with patient registers are a valuable resource for epidemiological research (1). The usefulness of DBSS is limited by the small amount of blood available, however, and methods that optimize the use of the sample are required, such as various whole genome amplification (WGA) methods used in genetic epidemiological studies (2, 3). We previously described a high-capacity multiplex immunoasay, based on Luminex xMAP technology that uses two 3-mm punches from each DBSS, for simultaneous determination of 25 inflammatory markers and neurotrophins (4). We now report that after extraction of proteins for the immunoassays, there is sufficient genomic DNA (gDNA) on the used DBSS disks to perform several WGA reactions, each producing enough whole-genome-amplified DNA (wgaDNA) for numerous reliable genotypings.

Blood from 20 anonymous volunteers was spotted on filter paper and air dried, and six 3-mm disks were punched out from each DBSS. Half the disks were subjected to protein extraction (4). The 2 groups of disks from each individual were then further split into 2 subgroups with, respectively, 1 and 2 disks in each, and gDNA was extracted in a volume of 200 μL using the Extract-N-Amp® reagent set (Sigma-Aldrich). The concentration of gDNA (0.2 mg/L, measured by the Quant-IT® PicoGreen® dsDNA Reagent) was not correlated to the use of 1 or 2 disks or to prior protein extraction. We then amplified extracted gDNA with 2 different WGA reagent sets: the GenomePlex Whole Genome Amplification Kit (Sigma-Aldrich), which is based on the Omniplex method, and the AmpliQ Genomic Amplifier Kit (Ampliqon), which uses the multiple-displacement amplification approach. Allowed input volumes are 10 μL for the GenomePlex (~2.0 ng gDNA) and 8 μL for the AmpliQ (~1.6 ng gDNA). Both reagent sets use less input gDNA than the recommended 10 ng, a feature that may be critical for the genotyping performance of the resulting wgaDNA (5), and thus direct comparisons cannot be made. The yield of wgaDNA was 2.6–3.8 μg, independent of whether protein extraction was performed and whether 1 or 2 disks were used.

The quality of wgaDNA was assessed by TaqMan® single-nucleotide polymorphism (SNP) genotyping of 27 SNPs in 27 genes distributed on 13 chromosomes. Reference gDNA was extracted from 200 μL of whole blood from the same 20 individuals. Genotype calls were...
done independently by 3 persons. The call and error rates are shown in Table 1. Despite the suboptimal amount of input gDNA, the best call and error rates were nearly optimal. The most common errors, results indicating that heterozygous individuals were homozygous, were attributable to allele dropouts most probably due to an insufficient amount or low quality of template gDNA. Details of sample flow and call and error rates for each individual gene can be seen in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue5.

When extraction of proteins from disks was performed before extraction of DNA, the resulting wgaDNA generally showed higher TaqMan genotype call rates and lower error rates than wgaDNA from disks that had not undergone pre-extraction of proteins (Table 1). This result suggests that the pre-extraction procedure removes compounds from the filter paper disks that may interfere with the WGA reaction. This explanation is in accordance with the observation that the use of 2 disks that had not undergone protein pre-extraction gave inferior results than did the use of only 1 disk, whereas the opposite was the case for the pre-extracted disks, for which the use of 2 disks instead of 1 yielded superior results (Table 1). The DBSS used in this study were stored for only a limited time, and we do not know the effect on the quality of wgaDNA of prolonged storage at room temperature or at −20°C.

Our results demonstrate that DBSS disks previously used for multiplex protein measurements are reliable sources of gDNA that is suitable for WGA and SNP genotyping. The study also shows that both the Omniplex-based method producing short wgaDNA of 400–500 bp and the multiple-displacement amplification-based method producing long wgaDNA of 10–20 kbp are well suited for the amplification. In our setup, the amount of template gDNA from each extraction was sufficient for ~20 amplifications, and the yield of wgaDNA from 1 amplification was enough for ~300 TaqMan genotypes.

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References


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Pyrosequencing Technology for Automated Detection of the BMP15 A180T Variant in Spanish Postmenopausal Women

To the Editor:

Germline mutations in different genes are associated with premature ovarian failure (POF, OMIM 311360), defined as premature menopause with amenorrhea occurring before the age of 40 years along with increased gonadotropin concentrations [follicle-stimulating hormone (FSH) >40 IU/L]. A new candidate gene, bone morphogenetic protein 15 (BMP15) has been investigated in POF. In a family affected by hypergonadotropic ovarian failure, a mutation in the pro-region of the BMP15 gene (Y235C) was found in 2 affected sisters (1), and 3 linked markers within the BMP15 gene (−673C>T, −9C>G and IVS1 + 905A>G) are associated with high follicle production in women undergoing recombinant FSH stimulation (2).

Several heterozygous variations affecting the pro-region and mature peptide of the BMP15 gene have been identified in women with POF (3–5), but A180T was the only variant found in all reported studies, occurring with relatively high frequency in POF women (8 of 502, 1.6%) but not at all in control groups.

To clarify the role of the A180T allele in early menopause and ovarian failure, we used a pyrosequencing protocol (Biotage) to evaluate the A180T variant. This technology allows an easy 96-well typing format. The selected primers for pyrose-
quencing analysis and PCR conditions were forward: 5'-ACC GCC ATC ATC TCC AAC TAA-3’ and reverse: 5’-biotine-CCT GTG TCC TGT ATT CCA-3’, and sequencing: 5’-AAC CCC TGA TGT CT-3’. We used reextraction and conventional resequencing of a random set of samples (10%) with the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman) to assure the quality of our genotyping protocol (see Figure 1 in the Data Supplement that accompanies the online version of this letter at http://www.clinchem.org/content/vol53/issue4).

We investigated the presence of the A180T allele and also reproductive, gynecological, and other important traits in 1157 white Spanish postmenopausal women divided into 3 clinical groups. Group A included women whose age at natural menopause was in the normal range (≥40 years, n = 852), group B included women with secondary amenorrhea (SA; age at natural menopause <40 years, n = 46), and group C included women with menopause induced by surgery for treatment of benign or malignant gynecological pathologies (n = 249). The observed mean (SD) ages at menopause and menarche [48.4 (5.0) and 12.9 (1.56) years, respectively] were strictly concordant with others previously reported. Written informed consent was obtained from all study participants, and the study protocol was approved by the referral center Ethics Committees and Neocodex.

Genotyping revealed the A180T variant only in 7 unrelated individuals, suggesting a variant allele frequency of 0.3% in the Spanish population. The clinical data and related morbidity of the 7 women who were heterozygous carriers of A180T variant are reported in Table 1. Among these women, 1 patient had menopause caused by a surgical intervention for endometriosis (group C). Only 1 patient displayed the SA phenotype (group B). The other 5 women had proven fertility and a natural menopause occurring at >40 years (group A).

To analyze relevant variables involved in age at menopause, such as age at menarche, reproductive period, pregnancies, and body mass index, we further studied patients with a natural menopause (n = 898) by dividing them into 2 genotypic groups (presence/absence of A180T variant). Our results indicated that the groups did not differ in any clinical variable studied (P >0.13). Nevertheless, women carrying the A180T variant seemed to have a lower mean (SD) age at menopause [45.3 (5.6) vs. 48.5 (5.0)], and they had a shorter reproductive period [32.8 (5.4) vs. 35.6 (5.1) years], although because of the small number of individuals, this finding was not statistically significant.

Table 1. Clinical profile of patients carrying BMP15 A180T variant.

<table>
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<th>Phenotypic Features</th>
<th>83</th>
<th>363</th>
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<th>477</th>
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<tr>
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<sup>a</sup> Reproductive period; time comprised between menarche and menopause.

Research to date has detected the A180T variant in only 10 women. Of these, 5 of 166 were Italian women with POF (3); 1 of 133 and 2 of 60 Indian women with POF or primary amenorrhea, respectively (4); and 2 of 203 European women with POF (5). Furthermore, no control individuals analyzed during date have been carriers the A180T allele (n = 462, merged sample size). In contrast with these observations, we detected the A180T allele in 5 women with natural menopause occurring at >40 years of age. Our results indicate that the A180T allele is a rare variant that is not sufficient to generate SA in humans. That the allele has not been found in control groups to date does not exclude the possible presence of this rare variant in unaffected individuals, because the number of controls used in studies to date may have been insufficient to detect very low frequency alleles. However, our data also support the finding that genetic variation in the BMP15 gene may contribute to variation in age at menopause in a complex manner, but this interpretation should be viewed with caution.

To elucidate the role of BMP15 in ovulation rate, POF, and SA, exhaustive analysis of this gene in independent large populations would be required.
We are deeply grateful to the post-menopausal women who participated in this study. We are very grateful to Eva Molero, Antonio González, and Rocío Pascual for their collaboration during this work. The authors F.J. Morón, M.E. Saez, and A. Ruiz, have declared that conflicts of interest exist. Some of the work described here is subject to patent filings for diagnostics purposes. Neocodex has been partially funded by the Ministerio de Educación y Ciencia of Spain (PTQ2003-0546, PTQ2003-0549, PTQ2003-0783) and the European Commission (Gendisrupt project: QLK4-CT-2002-02403).

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To the Editor:

Shipkova et al. (1) recently reported in Clinical Chemistry that liquid chroma- tography can be used to determine the activity of inosine triphosphate pyrophosphohydrolase (ITPase) in erythrocytes. We find that ITPase can also be measured by capillary electrophoresis.

We performed capillary electrophoresis on a Beckman P/ACE 5510 with a diode array detector. Electrophoretic separations were carried out in an uncoated silica capillary (20 cm effective/27 cm total length, 50 μm internal diameter; Polymicro CE and CEC Technologies) at a constant voltage of ~30 kV (1111 V/cm). We set the data rate of the detector at 16 Hz. Samples were loaded by a low-pressure injection (0.5 psi, 6 s). Ultra-violet detection was performed at 250 nm.

We prepared buffer containing citric acid (40 mmol/L) and cetyltrimethylammonium bromide (0.8 mmol/L), adjusted to pH 4.4 with γ-aminobutyric acid (2), filtered and sonicated for 0.5 min before use. At the beginning of each working day, the capillary was washed for 2 min with water and separation buffer, and also washed between runs for 1 min with separation buffer. We prepared a 100 μmol/L aqueous mixture of inosine monophosphate (IMP) and inosine triphosphate (ITP). The compounds could be separated within 0.8 min with separation efficiency up to 1 200 000 theoretical plates/m in a mixture and 300 000 theoretical plates/m in biological samples (Fig. 1).

We collected blood samples in EDTA tubes from healthy blood donors and patients undergoing azathioprine therapy (who gave in- formed consent). Erythrocytes were separated by centrifugation (1200g, 5 min) and washed twice with 3 volumes of NaCl, 9 g/L. We lysed 200 μL of erythrocytes with 1 mL of ice-cold distilled water, centrifuged the lysates at 5000g for 10 min, and mixed 25 μL of supernatant with 100 mmol/L Tris buffer (pH 9.0), 10 mmol/L dithiothreitol (10 μL), and 1 mol/L MgCl2 (10 μL). The mixture was preincubated for 5 min at 37 °C, after which 40 mmol/L ITP (10 μL) was added and incubated for 15 min at 37 °C (2). The samples were deproteinized with 20 μL of trichloroacetic acid, 1 mol/L, sonicated (30 s), and centrifuged at 5000g for 1 min. The supernatant was in- jected into the capillary or stored at ~50 °C. We measured hemoglobin (Hb) in the lysate with a RadiometerABL 725 (Diamond Diagnostics).

The signal-to-noise ratio was >6 at 2.0 nkat/g Hb [7.2 μmol of IMP/(g Hb · h)]. The calibration curve was linear from 0.01 to 10 mmol/L (y = 11.3x – 0.6 nkat/g Hb; r = 0.9974). We evaluated recovery and imprecis- ion by assaying erythrocytes with the added mixture of IMP. Recoveries were 85%, 83%, 76%, 75%, and 80% for 0.06, 0.25, 0.54, 1.80, and 3.00
mmol/L added IMP (n = 6), respectively. The lower recoveries reflect coprecipitation of IMP with proteins, which agrees with previously published findings (1). Imprecision values (as CV, n = 10) were 2.1%, 1.2%, and 1.0% (within-day CV) and 4.2%, 3.2%, and 2.4% (between-day CV) for 0.06, 0.54, and 3.00 mmol/L additions of IMP, respectively. The reproducibility values (CV) of migration times for 10 samples from healthy volunteers were 0.92%, 2.8%, and 2.5% for run-to-run, sample-to-sample, and between-day measurements (n = 10), respectively. Because of the use of acidic separation medium, we observed no interference during the analysis of samples from 80 healthy blood donors and 20 patients undergoing azathioprine therapy.

With this simple capillary electrophoresis method, we estimated a reference interval (n = 80, 38 males and 42 females) for healthy white individuals of 13.3–112.2 nkat/g Hb (5%–95%), with a median of 57.8 nkat/g Hb, which agrees with the previously published data (3).

Availability of alternative methods is important because available analytical equipment varies among laboratories. Measurements of ITPase are important because ITPase deficiency may alter 6-mercaptopurine (azathioprine) metabolism, leading to adverse reactions (3), and the deficiency affects >10% of the population (4).

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References


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Novel Mutation (c.G1124A) in Exon 9 of the APOB Gene Causes Aberrant Splicing and Familial Hypobetalipoproteinemia

To the Editor:

Familial hypobetalipoproteinemia (FHBL) is commonly caused by mutations in the apolipoprotein B gene (APOB). The APOB gene encodes 2 proteins, apolipoprotein (apo) B-48 and apo B-100. Apo B-48 is formed in the intestine and is essential for the formation and recognition of dietary derived chylomicrons, and apo B-100 is found in VLDLs and LDLs of hepatic origin and is involved in the endogenous transport of triglycerides, cholesterol, and fat-soluble vitamins. A number of abnormally truncated apo B proteins have been described, and by convention are referred to by a centile system reflect-
ing their apparent $M_r$ in relation to apo B-100 (1).

Truncations shorter than apo B-27 are not expressed in lipoproteins, and those shorter than apo B-75 are underrepresented in LDL (2–4). Consequently homozygous mutations in the N-terminal third of APOB result in the virtual absence of both apo B-48 and apo B-100 and their corresponding lipoproteins, and thus very low concentrations of plasma triglycerides, cholesterol, and the fat-soluble vitamins. This condition is known as FHBL and is characterized clinically by failure to thrive, steatorrhea, and eventually both central and peripheral neurological abnormalities (1). Heterozygotes usually experience a milder phenotype or are asymptomatic.

We report a novel APOB mutation, identified in a family with low total cholesterol and apo B concentrations in plasma. The proband, a 64-year-old man, had an LDL cholesterol concentration of 1.4 mmol/L and an apo B concentration of 0.39 g/L, and his 2 daughters both had LDL cholesterol concentrations $\leq$0.5 mmol/L, and apo B concentrations $<0.35$ g/L.

In the mother, the concentrations of these analytes were within reference intervals. Western blotting of plasma from all 4 individuals showed no apo B truncations. DNA sequencing of the exons and exon/intron boundaries of the APOB gene revealed a novel heterozygous c.G1124A mutation in the proband and his 2 daughters, which was not present in the mother. No other APOB gene mutations were identified.

The c.G1124A mutation predicts a p.Ser348Asn substitution in the $\beta\alpha_1$ domain, which is essential for lipoprotein assembly. The p.Ser348Asn substitution may affect the structure or function of this domain but is predicted to be benign, according to Polyphen (http://www.polypheb.com), with a position-specific independent counts difference score of 0.675. Alternatively the mutation at the ultimate nucleotide of exon 9 could affect splicing at the adjacent intron 9 donor splice site, with various potential splicing outcomes (Fig. 1A).

Indeed, the programs SpliceView (http://l25.itba.mi.cnr.it/~webgene/wwspliceview.html) and NNSplice (http://www.fruitfly.org/seq_tools/splice.html) predicted that the G$\rightarrow$A mutation would abolish splicing at the normal donor splice site of intron 9, and activate a cryptic donor site 40 bp into the intron. Gene Splicer (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html) also predicted abolishment of the normal donor site but did not predict the usage of a cryptic splice site.

To confirm these in silico predictions we performed minigene expression studies. A minigene construct spanning exons 8–11 and the intervening sequences was cloned into the pcDNA3.1/V5-His TOPO TA vector and then transfected into COS-7 cells. After 48 h the mRNA was isolated and reverse transcription PCR was performed. The cDNA was amplified using primers within exon 8 and exon 11. The expected 650-bp product was visualized in the wild-type, and a larger product of 690 bp was observed in the mutant (Fig. 1B). DNA sequencing of the 690-bp and 650-bp bands revealed that the increase in size of the mutant product reflected the inclusion of the first 40 bp of intron 9. A cryptic donor splice site between c.1124 + 40 and c.1124 + 41 was activated in the mutant construct, and the normal intron 9 acceptor site was used. Predictably, this message results in a frame shift in the translated protein, a substitution of serine 348 to lysine, and the insertion of 92 new amino acids before a premature stop is encountered at residue 440 (Ser348LysfsX93). The resulting mutant protein, a truncated apo B-9.7, would not be viable for lipoprotein formation. In vivo the majority of the transcripts would be expected to use the cryptic splice site in intron 9, creating the truncated apo B-9.7 and causing the observed FHBL.

Fig. 1. Pre-mRNA splicing of the G1124A minigene construct.
(A), diagram of the minigene construct and the potential splicing outcomes from the G1124A mutation. (B), 2% agarose gel showing the wild-type (WT) and mutant (MUT) cDNA products arising from splicing of the minigene construct in COS-7 cells. Two different-sized bands are visible at 650 and 690 bp, respectively.
From this analysis, we have shown that the novel c.G1124A mutation causes FHBL by disrupting splicing. We identified 3 family members who were heterozygous for this mutation but were largely asymptomatic because each still had 1 normal APOB allele.

This case highlights the difficulty of interpreting novel mutations identified in diagnostic laboratories and the need for a clear strategy to determine their significance. If sufficient family members are not available, linkage analysis may be uninformative and functional analysis is essential.

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References

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*Circulating Cell-Free Placental mRNA in the Maternal Plasma as a Predictive Marker for Twin-Twin Transfusion Syndrome

To the Editor:

Twin-twin transfusion syndrome (TTTS), which is a serious complication in monochorionic diamniotic twins (MCDA-T), involves unequal blood flow via the placental vascular anastomoses from the donor to the recipient twin. Although the placental anastomoses are present in all MCDA-T and both fetuses are genetically identical, TTTS occurs in only 15% of MCDA-T, and much of the pathophysiological basis of TTTS remains poorly understood. Clinically, a staging system based on the ultrasound features of TTTS is widely used for the management (1) but not for the prediction of TTTS. In addition, the known predictive findings observable by ultrasonographic examination are detectable only in a small portion of TTTS cases (2). New predictive markers are therefore desirable for the early detection and prevention of TTTS. Recently, placental mRNAs, such as human placental lactogen (PL) and some other hormones were detected in maternal plasma, and concentrations of each marker were measured with quantitative real-time reverse transcription (RT)-PCR assay (3, 4). Thus, circulating cell-free mRNA (cf-mRNA) in maternal plasma has become an attractive target for the noninvasive monitoring of pregnancy disorders (3, 5).

The purpose of the present study was to investigate the use of cf-mRNA concentration in maternal plasma as a predictive marker of later TTTS. The study participants included 17 pregnant women who visited the Obstetrics Clinic of Nagasaki University Hospital at 12–21 weeks of gestation for management of their pregnancy with MCDA-T. Included as a control group were 135 singleton pregnant women without medical complications at similar gestational age. All of the participants gave written informed consent, and the study was approved by the Research Ethics Committee of Nagasaki University. Although none of the 17 cases of MCDA-T were complicated by TTTS at the time of blood sampling, TTTS subsequently developed in 5 cases (TTTS group), but not in the remaining 12 cases (no-TTTS group). Gestational ages at diagnosis of TTTS were 15–25 weeks. The 3 groups had no significant differences in population characteristics, including the maternal age, the number of multiparous women, and the gestational age at the time of sampling (data not shown).

The blood samples (8 mL) from each woman were collected into an EDTA tube, and the plasma sample was stored at −20°C until use. After cf-mRNA was extracted from maternal plasma, a quantitative 1-step real-time RT-PCR assay was performed using an ABI 7900T Sequence Detector (Perkin-Elmer) as described previously (4). Primer sets and TaqMan probes for each gene and single-strand, and synthetic DNA oligonucleotides from each amplicon used for a calibration curve were prepared as described previously (4). Then, plasma concentrations of cf-mRNA for human PL and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured and converted into multiples of the median (MoM) of the controls adjusted for gestational age, as described previously (5). The differences between the TTTS and the no-TTTS groups were evaluated with the Mann–Whitney U-test. Significant difference was defined as a P value <0.05.

The median (minimum–maximum) cf-PL mRNA MoM values were 1.80 (0.89–3.81) in the TTTS group, 1.14 (0.77–1.35) in the no-TTTS group, and 1.00 (0.82–2.05) in the control group, respectively. At adjusted gestational age the cf-PL mRNA concentration was significantly higher in the TTTS group than in the no-TTTS group (Mann–Whitney U-test, P = 0.035), whereas there was no significant difference of cf-PL mRNA concentration between the no-TTTS group and the control group (P = 0.41; Fig. 1). In addition, the median cf-GAPDH mRNA MoM value in the maternal plasma was significantly higher in the TTTS...
group (2.20; range 1.30–2.68) than in the no-TTTS group (1.09; range 0.68–3.25; P = 0.045). Our results suggested the possibility that unapparent pathophysiological changes had already occurred in the women who subsequently developed TTTS, although which specific conditions led to the increased mRNA in the maternal plasma in the TTTS group remain unknown.

In conclusion, a quantitative aberration of both the cf-PL and cf-GAPDH mRNA in maternal circulation may be a novel predictive marker for TTTS, although both statistical differences were small and the sample size was too small to give sufficient strength to the analysis. Therefore, a combination of several cell-free placental mRNA markers could be effective for the prediction of TTTS, similar to the situation for tumor markers. Further study to identify gene transcripts that are expressed only in the placenta and not in blood cells may help to both predict and prevent TTTS and also may further elucidate the pathophysiology of this serious complication.

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Toenails: An Easily Accessible and Long-Term Stable Source of DNA for Genetic Analyses in Large-Scale Epidemiological Studies

To the Editor:
Molecular tools are increasingly applied in epidemiological studies to unravel the relationship between environmental exposures and disease (1). DNA is required for analyses of genetic factors, such as polymorphisms, but currently used specimens, such as lymphocytes and buccal cells, have disadvantages related to collection, transport, storage, and processing of samples. A relatively infrequently used source of DNA that may overcome these problems is nail material. Human toenails have been collected in several epidemiological studies, predominantly for determination of trace elements as biomarkers for the intake of these compounds (2). Until now, none of these epidemiological studies have applied human toenails as a source of DNA.

We investigated whether toenail material collected 20 years ago in the Netherlands Cohort Study on Diet and Cancer (NLCS) (3) (n = 120 852) could be used as a source of DNA for analyses of multiple genetic polymorphisms. Approximately 90 000 participants provided toenail clippings (on average, 80 mg per participant) (2). We optimized a protocol for DNA isolation from ~10 mg toenail material, based on the method of Cline et al. (4), and tested the suitability of this DNA in 2 PCR-based
assays in a subgroup of the cohort (n = 57) for which buccal DNA was also available. In the 1st assay, 10 single nucleotide polymorphisms were amplified in a multiplex PCR reaction and subsequently genotyped by means of single base extension using primer extension and automated capillary gel electrophoresis as described by Knaapen et al. (5). A 2nd PCR-based test was used to investigate the maximum length of fragments that could be amplified. A portion of the toenail samples had been irradiated with neutrons for analyses of selenium content (not irradiated: n = 11; irradiated: n = 24). To assess possible effects of age of the toenails on the quality of the DNA, freshly harvested toenail material from healthy non-NLCS volunteers was investigated (n = 11) (see the Data Supplement that accompanies the online version of this letter at http://www.clinchem.org/content/vol53/issue6 for a detailed description of material and methods).

DNA was successfully isolated from all toenail samples. On average, 2 µg DNA could be extracted from 10 mg toenail material (Table 1). There was no significant difference in DNA quantity between the various groups of toenail samples. The large range in DNA yield was mainly due to 2 outliers. The minimum amount isolated was almost 1 µg DNA/10 mg toenail material, which is sufficient for more than 10 multiplex genotyping analyses (80 ng of DNA is sufficient per analysis).

A summary of quantitative results of the multiplex genotyping assays on the DNA samples is provided in Table 1. A genotype profile of 10 polymorphisms was successfully generated for 100%, 96%, and 94% of the DNA samples isolated from fresh, 20-year-old irradiated, and 20-year-old not irradiated toenail material, respectively (amplicon sizes 92–148 bp). The success rate using the buccal swab DNA samples was 100%. Because DNA isolation was successful for only 90% of the buccal swab material in the NLCS samples, the use of nail material as a source of DNA resulted in a higher rate of successful outcomes. For 35 individuals, we compared the outcome of the genotyping assay for both their buccal swab DNA and toenail material DNA. Surprisingly, for 1 person, the 2 sources of DNA resulted in different genetic profiles. This result could not be related to technical errors, but may be from the switching or mislabeling of a sample during the collection process 20 years ago.

The NLCS study received approval by the Medical Ethical Committee of the University Hospital Maastricht and TNO Quality of Life, Zeist, The Netherlands. Study participants gave informed consent.

Results from the 2nd PCR test showed that DNA isolated from 20-year-old (not irradiated) toenail material or from fresh toenail material could be amplified to ≤596 bp. With DNA from 20-year-old irradiated nail material, generation of the 596-bp amplicon was unsuccessful for 60% of the samples, indicating increased fragmentation of the DNA. Irradiation probably causes fragmentation of the DNA, an effect that must be taken into account when the available nail material has previously been used for trace element analyses, which require irradiation.

In conclusion, we showed that 20-year-old nonirradiated and irradiated toenails can be a source of DNA for state of the art high-throughput genetic analyses of polymorphisms. For existing large-scale epidemiological studies, our results demonstrate that toenail material can be used for genetic analyses in cohorts for which no other source of DNA is available. The use of toenails as source of DNA may be of considerable relevance in future molecular epidemiological studies, because toenail clippings can be stored for long periods at low costs while DNA quality remains constant.

Grant/funding support: This study was supported by the Network of Excellence “Environmental Cancer Risk, Nutrition and Individual Susceptibility” (ECNIS), which operates in the context of the 6th European Union Framework Program for Research and Development (FP6). A.M.K. is supported by a postdoctoral fellowship from the Netherlands Organization for Scientific Research (NWO, VENI-Grant 916.46.092).

Table 1. DNA yield and multiplex genotyping results.

<table>
<thead>
<tr>
<th>Material*</th>
<th>Toenails</th>
<th>DNA yield, ng/10 mg toenail</th>
<th>Success rate of multiplex genotyping*</th>
<th>Corresponding buccal swabs available*</th>
<th>Genotype identical*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh material, &lt;3 months old</td>
<td>11</td>
<td>2102 (669)</td>
<td>11 (100%)</td>
<td>11 (100%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>NLCS material, 20 years old</td>
<td>24</td>
<td>2013 (1167)</td>
<td>23 (96%)</td>
<td>23</td>
<td>22 (96%)</td>
</tr>
<tr>
<td>Irradiated</td>
<td>33</td>
<td>2173 (1623)</td>
<td>31 (94%)</td>
<td>12</td>
<td>12 (100%)</td>
</tr>
<tr>
<td>Not irradiated</td>
<td>Total NLCS</td>
<td>57</td>
<td>2106 (1439)</td>
<td>54 (95%)</td>
<td>35</td>
</tr>
</tbody>
</table>

a Material: NLCS material, toenail material collected in the Netherlands Cohort study on Diet and Cancer; Irradiated: toenail material irradiated with neutrons to determine selenium content (Instrumental Neutron Activation Analysis).

b Number of toenail samples for which a comparison with buccal swab samples was feasible.

c Individuals for whom all 10 polymorphisms in the toenail sample were identical to those in the buccal swab sample.

d NA, Not applicable.
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Correction
The article by John Middleton and Jeffrey E. Vaks entitled “Evaluation of Assigned Value Uncertainty for Complex Calibrator Value Assignment Processes: A Prealbumin Example” (Clin Chem 2007;53:735–41), contains several errors. In the first section of Results, page 739, the text should be changed from “The IWC value assignment results are shown in Table 1” to “The IWC level 8 results are given in Table 1”. Also, in Table 2, for IWC5 to IWC8, the decimal point in the uncertainty SD’s should be shifted one digit to the left. Additionally, in Table 3, footnote b, the reference interval should be changed from “...based on width of 20 mg/L of the...18–38 mg/L” to “...based on width of 200 mg/L of the...180–380 mg/L.” Also, in Table 3, the superscript “a” should be removed from the 180 and 380 mg/L table entries. Finally, in the 4th paragraph in Results (page 739), the text should be changed from “...we made 48 measurements (4 instruments, 12 replicates per sample)....” to “...we made 96 measurements (4 instruments, 24 replicates per sample)....” The authors regret the errors.

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