Increased shedding of angiotensin-converting enzyme by a mutation identified in the stalk region. J Biol Chem 2001;276:5525–32.
21. Okabe T, Fujisawa M, Yotsumoto H, Watanabe J, Takaku F, Lanzillo JJ, et al. Familial elevation of serum angiotensin-converting enzyme. Q J Med 1985; 55:55–61.
22. Luisetti M, Martinetti M, Cuccia M, Dugoujon M, De Rose V, Peona V, et al. Familial elevation of serum angiotensin-converting enzyme activity. Eur Resp J 1990;3:441–6.
23. Kramers C, Deinum J. Increased serum activity of angiotensin-converting enzyme (ACE): indication of sarcoidosis? A Bayesian approach. Ned Tijdschr Geneeskd 2003;147:473–6.
24. Linneback M, Kesper K, Jeub M, Urbach H, Wullner U, Klockgether T, et al. Hereditary elevation of angiotensin-converting enzyme suggesting neurosarcoidosis. Neurology 2003;61:1819–20.
25. Balyasnikova IV, Metzger R, Sun Z-L, Franke FE, Berestetskaya YV, Chubb AJ, et al. Monoclonal antibodies 1B3 and 5C8 as probes for monitoring the nativity of the soluble angiotensin-converting enzyme. Hybridoma 2005;24: 14–26.
26. Danilov S, Jaspard E, Churakova T, Towbin H, Savoie F, Wei L, et al. Structure-function analysis of angiotensin I-converting enzyme using monoclonal antibodies. J Biol Chem 1994;269:26806–14.
27. Kost OA, Balyasnikova IV, Chemodanova EE, Nikolskaya II, Albrecht RFII, Danilov SM. Epitope-dependent blocking of the angiotensin-converting enzyme dimerization by monoclonal antibodies to the N-terminal domain of ACE: possible link of ACE dimerization and shedding from the cell surface. Biochemistry 2003;42:6965–76.
28. Balyasnikova IV, Woodman ZL, Albrecht RFII, Natesh R, Acharya KR, Sturrock ED, et al. The localization of an N-domain region of angiotensin-converting enzyme involved in the regulation of ectodomain shedding using monoclonal antibodies. J Proteome Res 2005;4:258–67.
29. Daniels LB, Glew RH. β-Glucosidase assays in the diagnosis of Gaucher's disease. Clin Chem 1982;28(Pt 1):569–77.
30. Pilliquod Y, Reinharz A, Roth M. Studies on the angiotensin-converting enzyme with different substrates. Biochim Biophys Acta 1970;206:136–42.
31. Friedland J, Silverstein E. A sensitive fluorometric assay for serum angiotensin-converting enzyme. Am J Pathol 1976;66:416–24.
32. Danilov SM, Savoie F, Lenoir B, Jeunemaitre X, Azizi M, Tarnow L, et al. Development of enzyme-linked immunoassays for human angiotensin I-converting enzyme suitable for large-scale studies. J Hypertens 1996;14:719–27.

DOI: 10.1373/clinchem.2004.045633

Combination of His-Tagged T4 Endonuclease VII with Microplate Array Diagonal Gel Electrophoresis for High-Throughput Mutation Scanning, *Matt J. Smith,[1] Gabriella Pante-de-Sousa,[1,2] Khalid K. Alharbi,[1] Xiao-he Chen,[1] Ian N.M. Day,[1*] and Keith R. Fox*[3] [[1] Human Genetics Division, School of Medicine, Southampton University Hospital, Southampton, UK; [2] Department of Physiology, Federal University of Para, Belem-Para, Brazil; [3] School of Biological Sciences, University of Southampton, Southampton, UK; * address correspondence to this author at: Human Genetics Division, Duthie Building (Mp808), School of Medicine, Southampton University Hospital, Tremona Road, Southampton SO16 6YD, UK; fax 44-(0)23-80794264, e-mail inmd@soton.ac.uk]

Various physical mutation-scanning methods have been developed to avoid unnecessary resequencing of long stretches of DNA *(1–6)*. Protein-based mutation-scanning techniques include enzymatic digestion [reviewed in Ref. *(7)*], protein binding to a DNA duplex, and direct analyses of the in vivo or in vitro gene product. One such enzyme is T4 endonuclease VII (endoVII), the product of gene 49 of bacteriophage T4 *(8)*. Radiolabel replacement with fluorescent tags has facilitated automated analysis *(9)*. EndoVII recognizes heteroduplex structural distortions, nicking 2–6 bp 3′ to the distortion, with efficiency dependent on sequence context *(10)* and mismatch type *(11)*. Perfectly matched DNA undergoes some background digestion, which produces a highly reproducible pattern *(12)*. Mutation detection sensitivity obtained with endoVII digestion was found to be similar to that for denaturing HPLC and direct sequencing *(13)*.

Microplate array diagonal gel electrophoresis (MADGE) *(14)* provides an open-faced 96-well gel format for polyacrylamide gels. Recently, nondenaturing 192-, 384-, and 768-well formats of MADGE for high-throughput checking of PCR and post-PCR reactions *(15)* have been developed. We have combined, in proof-of-principle experiments, the mismatch digestion properties of endoVII with the high-throughput capabilities of MADGE and a newly developed denaturing MADGE format to create a simple mutation-scanning technique that can screen ~1000 PCR samples during a single 35-min electrophoretic run.

Plasmid pRB210 (T4 endonuclease VII in pET11a) was a kind gift from Professor B. Kemper (Institute for Genetics, University of Cologne, Germany). The PCR primers used to amplify the endoVII gene from pRB210 were as follows: forward, 5′-GCGCCATATGATGTTATTGAC-3′; reverse, 5′-CAGCGGATCCTCATTTTAAACT-3′. After trimming was performed with *Bam*HI and *Nde*I (New England Biolabs), pETendoVII was generated by ligation into pET15b (Novagen). Expressed N-terminal His-tagged endoVII was then purified by affinity chromatography.

We used a single colony from pETendoVII-transfected BL21 (DE3) Gold cells (Stratagene) to inoculate a 1-L Luria broth culture containing 100 μg/L carbenicillin. After overnight culture at 30 °C, an identical fresh 500-mL passage was made, and at mid-log phase of growth (absorbance at 600 nm, 0.6–0.8), protein expression was induced by 1 mmol/L isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 2 h by centrifugation at 5000*g* for 10 min, and then lysed by sonication (10 cycles of 30 s on and 30 s off at a probe amplitude of 10–15 μm in a MSE Soniprep 150). Cell debris and intact cells were removed by centrifugation at 10 000*g* for 40 min. All steps were carried out at 4 °C. The cell lysate was passed through a Schleicher & Schuell 0.2 μm single-use filter.

EndoVII was purified by use of 1-mL HiTrap columns in conjunction with the ΔKTA™ FPLC™ chromatography system (Amersham Bioscience), according to the manufacturer's instructions. Protein purity was assessed by sodium dodecyl sulfate gel electrophoresis (Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue6/), enzyme activity was confirmed (without His-tag removal) with digests of synthetic heteroduplex substrates (data not shown), and protein quantification was by Bradford assay. Storage was in 50 mmol/L Tris-HCl (pH 8) with 1 mmol/L dithiothreitol and 500 mL/L glycerol at −80 °C.

All primers were from MWG-Biotech. Exon 3 from wild-type *LDLR* (GenBank accession no. Nm_000527) was PCR-amplified using primers LDLR-F (5′-GCCTCAGTGGGTCTTTCCTT-3′) and LDLR-R (5′-CCAGGACTCAGATAGGCTCAA-3′), respectively, with 6-carboxyfluorescein (FAM) and hexachloro-6-carboxyfluorescein (HEX) 5′ end labels for probe generation or without end labels for generating amplicon from genomic DNAs for testing. Jumpstart Taq polymerase (Sigma-Aldrich) was used to ensure the highest quality probe generation, but the thermal and ionic conditions for probe and test sample amplifications were otherwise identical and essentially as given by Whittall et al. *(16)*. Probe PCR parallel reactions from microplate wells were pooled and purified with Wizard PCR prep reagents (Promega). The same 220-bp PCR amplicon of *LDLR* exon 3 was generated (with unlabeled primers) from samples from 330 unrelated familial hypercholesterolemic individuals previously mutation scanned by single-strand conformation polymorphism (SSCP) analysis *(16)* and by meltMADGE *(17)*. Six previously defined heterozygotes, c.259T>G (p.W66G), c.266G>A (p.C68Y), c.269A>G (p.D69G), c.301G>A (p.E80K), c.301delG (p.E80fs), and c.313 + 1G>A (splice site), were examined. Initially samples known to contain 1 of the 6 mutations were used to test endoVII digestion and were analyzed by capillary electrophoresis on an ABI-310 instrument. Subsequently, 330 amplicons were screened blind with denaturing MADGE (below) as the analytical platform. All protocols were developed by M.J. Smith and were validated by independent use by G. Pante-de-Sousa and X. Chen.

To form the heteroduplexes, we mixed 2.5 μL of purified fluorescently labeled probe (representing an equivalent volume of original PCR) and 5.5 μL of unpurified test PCR amplicon, heated the mixture to 95 °C, and allowed it to cool to reform duplex DNA. For endoVII digestion, we used a 10-μL reaction volume containing 8 μL of probe/test mixture and 2 μL of 5× endoVII reaction mixture [250 mmol/L K_2HPO_4 (pH 6.5), 25 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol, and 0.1 g/L endoVII]. Phosphate ions have been shown to improve the efficiency of endoVII *(18)*. Digestions were for 20 min at 37 °C.

EndoVII reaction mixture (2.5 μL) was mixed with 12 μL of deionized formamide, denatured at 95 °C for 5 min, and then chilled on ice before capillary electrophoresis (Applied Biosystems 310 Genetic Analyzer).

For endoVII-MADGE, the reaction was terminated by addition of 3 μL of loading dye (10 mmol/L NaOH, 50 mmol/L EDTA, 800 mL/L formamide, 2.5 g/L bromphenol blue, and 2.5 g/L xylene cyanole FF). Samples were denatured by heating at 95 °C for 5 min and placed on ice until gel loading.

EndoVII digestion fragments were resolved on a 10% polyacrylamide denaturing MADGE gel containing 7 mol/L urea and 1× Tris-borate-EDTA buffer [90 mmol/L Tris-HCl (pH 8.3), 90 mmol/L boric acid, 2 mmol/L EDTA]. After sample loading, the gel was covered by a second glass plate. This plate–gel–plate sandwich was secured by rubber bands, and silicon rubber tubing was inserted along the long edge of the sandwich to prevent electrophoretic edge artifacts. The assembly was placed in a purpose-built 2-L gel tank *(19)* (with capacity for 10 gels) containing 1× Tris-borate-EDTA buffer at 65 °C for electrophoresis at 10 V/cm for 35min. EndoVII-MADGE gels were scanned and analyzed with either a FluorImager™ 595 or a Typhoon Trio+ (Molecular Dynamics, Amersham Biosciences) and ImageQuant fragment analysis software (Molecular Dynamics).

LDLR mutations c.259T>G, c.301delG, c.301G>A, and c.313 + 1G>A generated a strong digest fragment for at least 1 probe strand, whereas c.266G>A generated a lower yield of digest fragment on 1 of the probe strands. c.269A>G displayed cleavage of the A·C heteroduplex when the label was on the C strand (mutant as probe). A typical example of the digestion pattern of the *LDLR* mutants can be seen in Fig. 2 of the online Data Supplement. The extra peaks observed corresponded to expected digest fragment sizes. These same products were trialed under various conditions in denaturing MADGE gels followed by fluoroimaging: the protocol described above was efficient.

A typical 96-well endoVII-MADGE gel from blind scanning of 330 familial hypercholesterolemic individuals is shown in Fig. 1 (also shown, with dual label, in Fig. 3 of the online Data Supplement). Previous mutation scanning of this sample set had identified 47 heterozygous individuals with 1 of the 6 mutations: c.259T>G, c.266G>A, c.269A>G, c.301G>A, c.301delG, or c.313 + 1G>A (Table 1). When we used only wild-type probe, endoVII-MADGE identified 51 samples containing additional digest fragments; 46 of these corresponded to the previously identified mutations covering 5 of the 6 known *LDLR* mutations (c.259T>G, c.266G>A, c.301G>A, c.301delG, and c.313 + 1G>A). The c.269A>G mutation remained undetected (see above). Of the 5 additional samples, 3 displayed digestion patterns matching those for positively identified known *LDLR* mutations: 1 with the pattern for c.259T>G and 2 with the pattern for c.301G>A. The remaining 2 samples displayed unique digest patterns that did not correspond to digest patterns for the 5 known mutations (Fig. 4A in the online Data Supplement). One digest pattern was similar to that for c.313 + 1G>A, but c.313 + 1G>A was characterized by a strong digestion fragment, whereas the unidentified mutation produced a significantly weaker fragment (Fig. 4B in the online Data Supplement). Sequencing showed that the sample was heterozygous for the base change c.311G>T. The second sample produced a digestion fragment close to the undigested amplicon. Sequencing showed a 2-base deletion, c.196_197delGT. c.311G>T has been reported previously (www.ucl.ac.uk/fh/genebook.html), whereas c.196_197delGT appears to be a novel mutation. Of the 7 mutations detected, 2 displayed detectable mismatch-specific digestion patterns in both the sense and antisense strands, c.196_197delGT and c.259T>G, whereas the remainder were identified by digestion of 1 strand.

This study suggests the feasibility of combining the

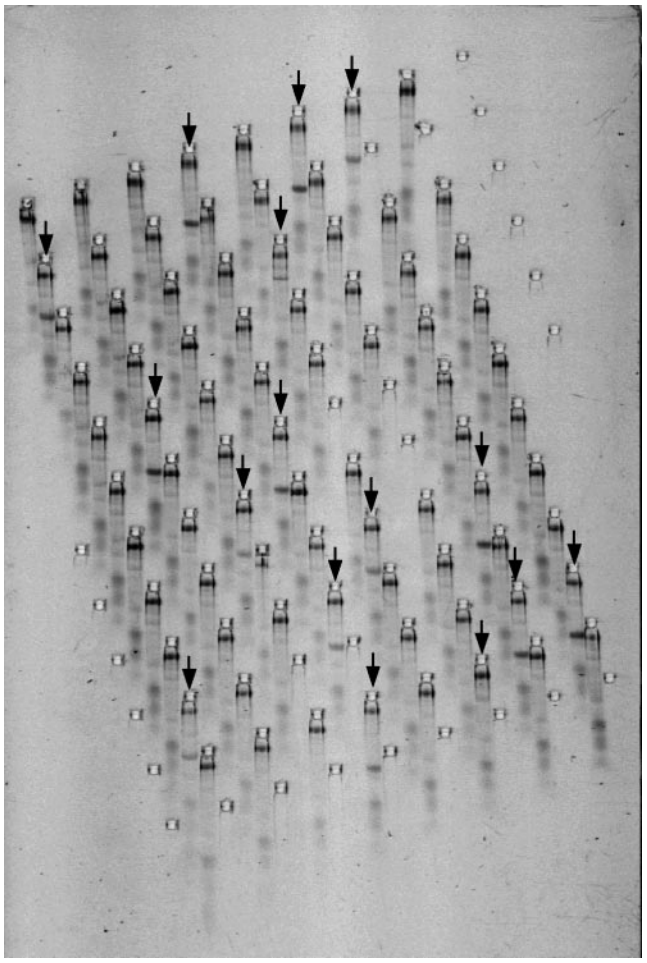

Fig. 1. EndoVII-MADGE analysis.

Shown is a typical endoVII-MADGE gel image for the *LDLR* exon 3 amplicon. The 8 × 12 array set at a 71.6-degree angle allows tracks to pass through 2 successive rows, allowing 96 samples to be run on a single gel. Samples containing mutations (tracks indicated by *arrows*) were identified by the presence of an extra band or by an increase in intensity of a background band. In this example, the 5′ fluorescent label was on the antisense strand.

mismatch digestion properties of endoVII with the high-throughput capabilities of MADGE to create a simple high-throughput mutation-scanning method. We found that the reduced resolution and increased relative background associated with short-track electrophoresis did not decrease the rate of mutation detection. EndoVII-MADGE also identified 2 previously unrecognized mutations in the sample set. EndoVII-MADGE consistently compared favorably with SSCP analysis of the same region (Table 1) in many heterozygotes, detecting 7 of 8 different sequence variations (8 of 8 when test samples were end labeled). This approach could potentially add to strategies for the investigation of unknown mutations at the population level.

Table 1. Number of separate cases detected for a set of mutations distributed through *LDLR* exon 3.

		No. of cases detected	
Mutation	**Amino acid change**	**SSCP analysis**	**EndoVII-MADGE with wild-type probe**
c.313 + 1G>A	Splice site	21	21
c.311G>T	p.C83F	0	1
c.301G>A	p.E80K	17	19
c.301delG	p.E80fs	3	3
c.269A>G	p.D69G	1	0
c.266G>A	p.C68Y	2	2
c.259T>G	p.W66G	3	4
c.196_197delGT	p.V45fs	0	1
Wild type		283	279

M.J. Smith was the recipient of a University of Southampton Faculty of Health Medicine and Life Science cross-school PhD studentship. We thank Professor Borries Kemper for clone pRB210. This work was also supported by the UK Department of Health, National Genetics Reference Laboratory (Wessex), and HOPE.

References

1. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A 1989;86:2766–70.
2. Fischer SG, Lerman LS. Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. Cell 1979;16:191–200.
3. Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, et al. Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 1997;7:996–1005.
4. Cotton RG, Rodrigues NR, Campbell RD. Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc Natl Acad Sci U S A 1988;85:4397–401.
5. Li Q, Liu Z, Monroe H, Culiat CT. Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. Electrophoresis 2002;23:1499–511.
6. Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin Chem 2004; 50:1748–54.
7. Taylor GR. Enzymatic and chemical cleavage methods. Electrophoresis 1999;20:1125–30.
8. Youil R, Kemper BW, Cotton RG. Screening for mutations by enzyme mismatch cleavage with T4 endonuclease VII. Proc Natl Acad Sci U S A 1995;92:87–91.
9. Del Tito BJ, Poff HE 3rd, Novotny MA, Cartledge DM, Walker RI, Earl CD, et al. Automated fluorescent analysis procedure for enzymatic mutation detection. Clin Chem 1998;44:731–9.
10. Pottmeyer S, Kemper B. T4 endonuclease VII resolves cruciform DNA with nick and counter-nick and its activity is directed by local nucleotide sequence. J Mol Biol 1992;223:607–15.
11. Solaro PC, Birkenkamp K, Pfeiffer P, Kemper B. Endonuclease VII of phage T4 triggers mismatch correction in vitro. J Mol Biol 1993;230:868–77.
12. Youil R, Kemper B, Cotton RG. Detection of 81 of 81 known mouse β-globin promoter mutations with T4 endonuclease VII—the EMC method. Genomics 1996;32:431–5.
13. Andrulis IL, Anton-Culver H, Beck J, Bove B, Boyd J, Buys S, et al. Comparison of DNA- and RNA-based methods for detection of truncating BRCA1 mutations. Hum Mutat 2002;20:65–73.
14. Day IN, Humphries SE. Electrophoresis for genotyping: microtiter array diagonal gel electrophoresis on horizontal polyacrylamide gels, hydrolink, or agarose. Anal Biochem 1994;222:389–95.
15. Gaunt TR, Hinks LJ, Rassoulian H, Day IN. Manual 768 or 384 well microplate gel 'dry' electrophoresis for PCR checking and SNP genotyping. Nucleic Acids Res 2003;31:e48.
16. Whittall R, Gudnason V, Weavind GP, Day LB, Humphries SE, Day IN. Utilities for high throughput use of the single strand conformational polymorphism method: screening of 791 patients with familial hypercholesterolaemia for mutations in exon 3 of the low density lipoprotein receptor gene. J Med Genet 1995;32:509–15.
17. Day IN, Alharbi KK, Haddad I, Ye S, Lawlor DA, Whittal RA, et al. Mutation scanning of LDLR in the whole population: severe, moderate and silent mutations, paucimorphisms and cholesterol level. Eur J Hum Genet 2004; 12:302.

18. Golz S, Greger B, Kemper B. Enzymatic mutation detection. Phosphate ions increase incision efficiency of endonuclease VII at a variety of damage sites in DNA. Mutat Res 1998;382:85–92.

19. Day IN, O'Dell SD, Cash ID, Humphries SE, Weavind GP. Electrophoresis for genotyping: temporal thermal gradient gel electrophoresis for profiling of oligonucleotide dissociation. Nucleic Acids Res 1995;23:2404–12.

Previously published online at DOI: 10.1373/clinchem.2004.046755

Carbohydrate-Deficient Transferrin Measured by Capillary Zone Electrophoresis and by Turbidimetric Immunoassay for Identification of Young Heavy Drinkers,

Jean-Bernard Daeppen,[1] Frederic Anex,[1] Bernard Favrat,[2] Alvine Bissery,[1] Joelle Leutwyler,[1] Roland Gammeter,[1] Patrice Mangin,[2] and Marc Augsburger[2]* ([1] Alcohol Treatment Center, CHUV, Lausanne, Switzerland; [2] Institute of Forensic Medicine, CHUV, Lausanne, Switzerland; * address correspondence to this author at: Alcohol Treatment Center, Mont-Paisible 16, CHUV, 1011 Lausanne, Switzerland; e-mail jean-bernard.daeppen@inst.hospvd.ch)

Carbohydrate-deficient transferrin (CDT) measured by capillary zone electrophoresis (CZE), particularly asialotransferrin (Tf), is purported to better differentiate between excessive and moderate drinkers than does CDT measured by turbidimetric immunoassay (TIA) *(1, 2)*. The use of biological markers such as CDT is of particular interest for identifying young heavy drinkers because other clinical signs of heavy drinking are generally absent and heavy drinking is a leading cause of morbidity and mortality in this age group *(3, 4)*. Several authors have shown interest in the ability of CDT to identify nondependent heavy drinkers *(5, 6)*; we therefore describe here the performance of CZE measurements of asialo- and disialo-Tf and TIA analysis of CDT in a large community sample of 19-year-old men, of whom 21% were heavy drinkers.

From a sample of 1018 men attending a mandatory 1-day army recruitment process for all Swiss males at age 19 years, 1004 (98.6%) agreed to complete a research questionnaire. Of these, 581 young men (57.9%) consented to give blood for the measurement of asialo-Tf (CZE), disialo-Tf (CZE), and CDT (TIA). The Ethics Committee of the Lausanne University Medical School approved the study protocol. Volunteers were compensated for participation in the study.

Volunters gave written informed consent and then completed an instrument entitled "Health and Lifestyle Questionnaire", which included questions assessing the typical quantity and frequency of alcohol consumption during the 12 months preceding the survey and the frequency of drunkenness over the last 30 days. One drink was defined as a 250-mL can or bottle of beer, a 120-mL glass of wine, or a 40-mL shot of liquor straight or in a mixed drink, and corresponded to ~12 g of pure ethanol.

A study investigator was present during administration of the questionnaire to verify that participants answered all items. Serum samples were obtained by centrifugation of peripheral blood collected in 10-mL tubes. Samples were stored at −20 °C before analysis.

Total CDT was measured by anion-exchange chromatography and TIA with the Axis-Shield CDT (TIA) reagent set *(7)*. To separate and measure Tf isoforms, we used a previously described and validated CZE method *(8, 9)* with the Ceofix CDT reagent set (Analis) on a Hewlett Packard (HP) 3D-CE instrument. The CZE conditions are described in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue6/. CZE electropherograms showing the serum Tf profiles for a heavy drinker before and after addition of anti-Tf polyclonal antibody to the serum are shown in Fig. 1 of the online Data Supplement, and CZE electropherograms showing the Tf profiles of a teetotaler and of 2 heavy drinkers are shown in Fig. 2 of the online Data Supplement.

Peaks representing the different Tf isoforms were quantified as the amounts of the asialo-, disialo-, trisialo-, tetrasialo-, pentasialo-, and hexasialo-Tf (CZE) as a percentage of the total Tf content, in terms of valley-to-valley areas under the curve. The intraday CV values (n = 6) for "low" (0.6% by CZE) and "high" disialo-Tf (4.8% by CZE) were 9.8% and 1.2%, respectively, and the interday CVs (n = 5) for low (0.6% by CZE) and high disialo-Tf (4.8% by CZE) were 11% and 2.3%, respectively. The intra- and interday CVs for asialo-Tf (0.5% by CZE; n = 6) were 6.8% and 11%, respectively. The limit of quantification of each Tf (CZE) isoform was 0.1%, expressed a percentage of total Tf isoforms.

Continuous data are reported as the mean (SD) and the median (interquartile range). We used a χ^2 test to compare categorical variables and Mann–Whitney *U*-tests to compare continuous variables because this nonparametric statistic makes no assumption about the distributional properties of variables. We also determined the areas under the ROC curves (AUROC), the sensitivity, and the specificity for disialo-Tf (measured by CZE) and CDT (measured by TIA) in identifying heavy drinkers.

There were 121 (20.8%) heavy drinkers in the sample: 31 (5.3%) who reported typical alcohol consumption of >21 drinks/week over the last 12 months, 52 (8.9%) who said they had been drunk at least 3 times over the last month; and 38 (6.5%) who reported both. Mean (SD) alcohol consumption in heavy drinkers was 26.4 (8.4) drinks (~300 g of ethanol) per week. Among the remaining participants, 435 (74.9%) were categorized as moderate drinkers, reporting, on average, 6.0 (4.7) drinks (~65 g of ethanol) per week, and 25 (4.3%) were considered abstinent (mean reported quantity and frequency = 0). The abstaining participants were retained as part of the moderate-drinker group.

Our results indicate that asialo-Tf (CZE) could not differentiate between moderate and heavy drinkers because 574 (98.8%) of the participants had a asialo-Tf (CZE) value of 0% and only 3 moderate drinkers and 4 heavy drinkers had positive values. We did, however, find significant differences between heavy and moderate