

**Postcollection Synthesis of Ethyl Glucuronide by Bacteria in Urine May Cause False Identification of Alcohol Consumption**, Anders Helander,\* Ingrid Olsson, and Helen Dahl (Department of Clinical Neuroscience, Karolinska Institute and Karolinska University Hospital, Stockholm, Sweden; \* address correspondence to this author at: Alcohol Laboratory, L7:03, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden; fax 46-8-51771532, e-mail anders.helander@ki.se)

**Background:** Ethyl glucuronide (EtG) is a minor ethanol metabolite used as a specific marker to document recent alcohol consumption; confirm abstinence in treatment programs, workplaces, and schools; and provide legal proof of drinking. This study examined if bacterial pathogens in urine may enable postsampling synthesis of EtG and ethyl sulfate (EtS) from ethanol, leading to clinical false-positive results.

**Methods:** Urine specimens with confirmed growth of *Escherichia coli*, *Klebsiella pneumoniae*, or *Enterobacter cloacae* were stored at room temperature in the presence of ethanol. Ethanol was either added to the samples or generated by inoculation with the fermenting yeast species *Candida albicans* and glucose as substrate. EtG and EtS were measured by LC-MS.

**Results:** High concentrations of EtG (24-h range 0.5–17.6 mg/L) were produced during storage in 35% of *E. coli*-infected urines containing ethanol. In some specimens that were initially EtG positive because of recent alcohol consumption, EtG was also sensitive to degradation by bacterial hydrolysis. In contrast, EtS was completely stable under these conditions.

**Conclusions:** The presence of EtG in urine is not a unique indicator of recent drinking, but might originate from postcollection synthesis if specimens are infected with *E. coli* and contain ethanol. Given the associated risks for false identification of alcohol consumption and false-negative EtG results due to bacterial degradation, we recommend that measurement of EtG be combined with EtS, or in the future possibly replaced by EtS.

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Early recognition of problem drinking or relapse is important to ensure adequate alcohol treatment strategies (1). This goal has been hampered by a lack of sufficiently sensitive and specific diagnostic methods. The reliability of self-reporting is limited by denial and underreporting (2). The time frame for identifying alcohol use by ethanol testing is usually limited to <12 h, because of rapid metabolism and excretion (3). Research has therefore focused on developing alcohol biomarkers with a longer detection window (4).

A new laboratory marker for detecting recent alcohol consumption is ethyl glucuronide (EtG) (5). EtG and ethyl sulfate (EtS) (6) are minor ethanol metabolites formed by uridine diphosphate–glucuronosyltransferase (UGT) and sulfotransferase (SULT), respectively, and excreted in

urine for a longer time than ethanol (7–10). Positive EtG and/or EtS test results thus provide a strong indication that the person has recently consumed alcohol, even when ethanol is no longer detectable (9). LC-MS methods are available for EtG and EtS detection (6, 10), as is an enzyme immunoassay for EtG (DRI<sup>®</sup> EtG, Microgenics).

EtG has been recommended for forensic application (11–13) and is used for documentation of abstinence in treatment programs, for alcohol testing in the workplace and schools, and as legal proof of drinking (known as “the 80-h alcohol test”). However, the high diagnostic sensitivity of the EtG test has also produced adverse publicity (14), because unintentional ethanol intake from ethanol-based mouthwash (15) and hand sanitizers (16) may also generate positive results. The United States Substance Abuse and Mental Health Services Administration recently warned against using a positive EtG as primary or sole evidence of drinking for disciplinary and legal action (17).

Bacterial contamination of urine may cause false-negative EtG test results (18). Many strains of *Escherichia coli*, the main source of urinary tract infections, contain the enzyme  $\beta$ -glucuronidase, which hydrolyzes EtG. Given that UGT and SULT activity also occur with some bacteria (19, 20), we examined whether human pathogens may enable postcollection synthesis of EtG and EtS from ethanol in urine.

Fresh human urine specimens (anonymous surplus volumes) with confirmed growth of common pathogenic bacteria (*E. coli*, n = 36; *Klebsiella pneumoniae*, n = 6; *Enterobacter cloacae*, n = 6), as identified by culture on standard solid media, were used (study approved by the local ethics committee). The samples had been submitted for routine diagnostic testing in the Department of Clinical Microbiology, Karolinska University Hospital, and were stored refrigerated until use.

In the 1st experiment we added ethanol (final concentration 1.0 g/L) to urine samples and split them into tubes that were capped and stored at 4 °C and 22 °C. The same samples without addition of ethanol, or supplemented with ethanol and 10 g/L sodium fluoride as preservative, and uninfected urines served as controls. In the 2nd experiment ethanol was generated in the urine samples by inoculation with the fermenting yeast species *Candida albicans* (1 000 000 colony-forming units/L) and 20 g/L glucose as substrate. At the start of the experiment, and after different storage times at 4 °C and 22 °C, aliquots were stored at –20 °C before analysis of EtG, EtS, and ethanol.

EtG and EtS were quantified by an LC-MS method (6, 9, 10). Analysis was performed in the negative-ion mode using selected ion monitoring of the deprotonated ions at  $m/z$  125 for EtS and  $m/z$  130 for EtS-D5, and at  $m/z$  221 and  $m/z$  226 for EtG and EtG-D5. We purchased EtS from TCI and EtG and EtG-D5 from Medichem Diagnostics. EtS-D5 was synthesized (9). The previously determined detection limit was 0.1 mg/L; the routine clinical cutoff for EtG used in our laboratory is 0.5 mg/L. All positive EtG results by LC-MS were confirmed by LC-

tandem MS (Perkin–Elmer 200 LC and Sciex API 2000 MS) by the presence of the correct relative abundance of the major product ions of EtG ( $m/z$  75, 85, and 113). No interference by ion suppression was noted.

The ethanol concentration was determined enzymatically using alcohol dehydrogenase on a Hitachi 917 analyzer.

Of the 36 urine specimens infected by *E. coli*, 10 were positive for EtG (range 2.6–135.9 mg/L, mean 25.7 mg/L, median 10.4 mg/L) and EtS (range 1.3–20.0 mg/L, mean 5.0 mg/L, median 3.5 mg/L) at the start of the experiment, indicating that these patients had recently consumed alcohol. After these 10 samples were stored for 5 days at 22 °C, EtG was no longer detectable in 5 (50%), whereas the EtS concentrations remained unchanged. A disappearance of EtG, but not of EtS, was also observed after the samples had been supplemented with ethanol (Table 1). In 3 samples that initially contained 11.7–46.6 mg/L EtG, the concentrations were below the routine

clinical cutoff (<0.5 mg/L) after 24-h storage at 22 °C. These samples also showed a gradual disappearance of EtG at 4 °C, albeit at a much slower rate, whereas sodium fluoride was effective in preventing EtG degradation both at 4 °C and 22 °C (data not shown).

In 9 (35%) of the 26 urine specimens with confirmed growth of *E. coli* that were initially negative for EtG and EtS, formation of EtG but not of EtS was observed with time at 22 °C after addition of 1 g/L ethanol. In 7 samples (Fig. 1A), EtG concentrations above the clinical cutoff were observed after 24-h storage (range 0.5–17.6 mg/L, mean 5.2 mg/L, median 3.3 mg/L), and after 5 days the concentrations ranged from 0.3 to 35.2 mg/L (mean 8.9 mg/L, median 2.4 mg/L,  $n = 9$ ). Slow formation of EtG was also observed in 3 samples in the presence of added sodium fluoride and in 2 samples stored at 4 °C. After addition of ethanol to 1 urine specimen that initially contained 8.9 mg/L EtG and 2.0 mg/L EtS, the EtG concentration first increased to 17.6 mg/L after 24-h storage but then decreased to 5.6 mg/L after 48 h and then to <0.5 mg/L after 5 days (Fig. 1B). The corresponding EtS concentrations were stable at all times.

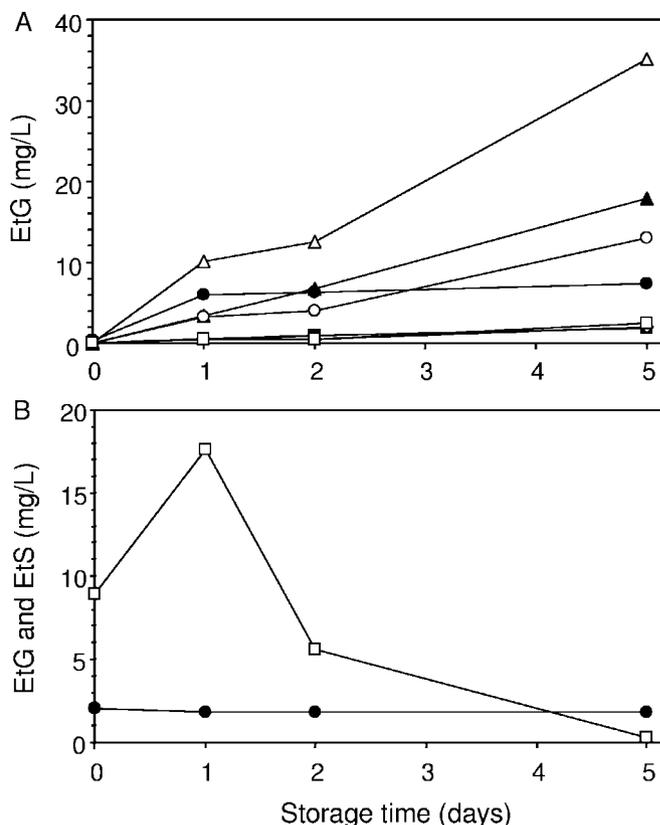
**Table 1. Stability of the minor ethanol metabolites EtG and EtS during storage of infected urine specimens.**

Test conditions	Uropathogen identified		
	<i>E. coli</i> , n	<i>K. pneumoniae</i> , n	<i>E. cloacae</i> , n
Infected urine specimens incubated with 1.0 g/L ethanol for 5 days <sup>a</sup>	36	6	6
Specimens initially positive for EtG and EtS <sup>b</sup>	10	1	4
EtG unchanged after 5 days	5	1	4
EtG negative or decreased after 5 days	5	0	0
EtS unchanged after 5 days	10	1	4
EtS negative or decreased after 5 days	0	0	0
Specimens initially negative for EtG and EtS	26	5	2
EtG negative after 5 days	17	5	2
EtG positive after 5 days <sup>c</sup>	9	0	0
EtS negative after 5 days	26	5	2
EtS positive after 5 days	0	0	0

<sup>a</sup> Ethanol was added to fresh urine specimens with confirmed growth of *E. coli*, *K. pneumoniae*, or *E. cloacae* and stored in sealed plastic vials without preservative at 22 °C for 5 days.

<sup>b</sup> Samples initially positive for EtG and EtS indicated that these patients had recently consumed alcohol. EtG and EtS were measured by LC-MS and the detection limit was approximately 0.1 mg/L for both compounds.

<sup>c</sup> All positive LC-MS results were confirmed positive by liquid chromatography-tandem mass spectrometry.



**Fig. 1.** Formation of the ethanol metabolite EtG in *E. coli* infected urine samples after addition of ethanol.

(A), urine specimens with confirmed growth of *E. coli* were supplemented with 1.0 g/L ethanol and stored at 22 °C. Individual EtG results for 7 urine samples are indicated by different symbols (the data for 3 samples that produced low concentrations of EtG partly overlap). (B), results for 1 urine specimen with confirmed growth of *E. coli* that showed both synthesis and degradation of EtG. The sample was initially positive for EtG (□) and EtS (●) and showed variable EtG but unchanged EtS concentrations with time after addition of ethanol and storage at 22 °C.

After 7 urine specimens containing *E. coli* and 5 uninfected control urines were supplemented with *C. albicans* and glucose to generate ethanol (all samples were initially negative for ethanol), the ethanol concentrations after 7-day storage at 22 °C ranged from 0.73 to 1.47 g/L (median 1.17 g/L). Formation of EtG (range 1.8–71.4 mg/L) was observed in 3 specimens containing *E. coli*, but in none of the uninfected controls. No formation of EtS was detected in these experiments.

No disappearance or formation of EtG or EtS was observed in the 12 urine specimens with confirmed growth of *K. pneumoniae* or *E. cloacae* after addition of ethanol or *C. albicans* and glucose and storage at 22 °C for 5 days (Table 1).

EtG has been considered specific for alcohol consumption and detectable only after *in vivo* ethanol metabolism, and hence EtG testing is used as a basis for disciplinary and legal action (17) and in forensic autopsy cases (13). A recent debate relates to the excellent analytical sensitivity of this test that, in combination with a low clinical cutoff concentration, may cause positive results attributable to unintentional ethanol exposure (14–16). To the best of our knowledge, no true false-positive EtG result has been reported without such exposure. Nonetheless, the present study demonstrated that EtG could be formed in a biological specimen after collection, if the specimen is infected with *E. coli* and ethanol is present or produced during storage. In our tested samples the formation of EtG was rapid and was not always prevented by addition of sodium fluoride or storage at refrigerator temperature.

Bacterial and fungal infections are common in clinical practice, with *E. coli* being the primary pathogen responsible for urinary tract infections. Ethanol may be formed in unpreserved biological specimens because of microbial contamination and fermentation, and this risk is especially high in diabetic patients as a result of glycosuria. Accordingly, considering the potential serious disciplinary and legal consequences if an individual is falsely accused of alcohol consumption on the basis of an incorrect EtG result, caution is advised when interpreting EtG test results, and the risk for postcollection ethanol formation must be considered.

The results of our study also confirm previous observations that EtG is sensitive to bacterial hydrolysis, but EtS is not (18). Accordingly, in situations in which EtG-positive urine is infected from the start, or becomes contaminated during handling, there is a risk for false-negative results and alcohol use will remain undetected.

The lack of EtS formation or degradation detected under the test conditions and the similar detection windows and sensitivities for recent alcohol consumption observed for the unique ethanol metabolites EtG and EtS (9) indicate that EtS testing should accompany, be used to verify, or in the future possibly replace EtG testing. The results further indicate that EtS is a more suitable test than EtG to distinguish antemortem ingestion of ethanol from postmortem synthesis in forensic toxicological analysis (13). Mass spectrometric methods for EtG can easily be modified to also quantify EtS (6, 9). If the analysis initially

focuses solely on EtG, EtS may be introduced as a verification assay. However, a false-negative EtG screening result will usually not be followed up with confirmatory analysis, and drinking will thereby remain undetected.

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**Stability of Soluble Adhesion Molecules, Selectins, and C-Reactive Protein at Various Temperatures: Implications for Epidemiological and Large-Scale Clinical Studies,** *Janine Hartweg,<sup>1,2\*</sup> Michael Gunter,<sup>1</sup> Rafael Perera,<sup>2</sup> Andrew Farmer,<sup>1,2</sup> Carole Cull,<sup>1†</sup> Casper Schalkwijk,<sup>3‡</sup> Astrid Kok,<sup>3</sup> Harry Twaalfhoven,<sup>3</sup> Rury Holman,<sup>1</sup> and Andrew Neil<sup>1,2</sup>* (<sup>1</sup> Diabetes Trials Unit, Oxford Centre for Diabetes, Endocrinology and Medicine, University of Oxford, United Kingdom; <sup>2</sup> Division of Public Health and Primary Health Care, University of Oxford, United Kingdom; <sup>3</sup> Clinical Chemistry, Institute for Cardiovascular Research VU University Medical Centre, Amsterdam, The Netherlands; † Dr. Carole Cull died in June 2007; ‡ current address: Department of Internal Medicine, University Hospital Maastricht, Maastricht, The Netherlands; \* address correspondence to this author at: Diabetes Trials Unit, Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford, OX3 7LJ, United Kingdom; fax 44 (0)1865 857240, e-mail janine.hartweg@ndm.ox.ac.uk)

**Background:** We assessed the impact of sample storage conditions on soluble vascular cell adhesion molecules (sVCAM), soluble intracellular adhesion molecules (sICAM-1), soluble (s)E-selectin, C-reactive protein (CRP), and sP-selectin.

**Methods:** Markers were measured by ELISA in venous blood from 10 healthy volunteers on aliquots stored as plasma or whole blood at 4, 21, or 30 °C for 1–5 days and after 1–5 freeze-thaw cycles. We compared results on these samples to results for samples processed immediately and stored at –80 °C. Statistical models assessed time-related effects and effects of postprocessing conditions.

**Results:** Using an upper limit of 10% variation from baseline with  $P > 0.05$ , we found that stability duration in plasma was 5 days for sVCAM-1 and sICAM-1 and at least 2 days for sE-selectin at 4, 21, and 30 °C and 5 days for CRP at 4 and 21 °C and 1 day at 30 °C. Stability duration in whole blood was 5 days for sVCAM-1 and sICAM-1 and at least 2 days for sE-selectin at 4, 21, and 30 °C and 5 days for CRP at 4 and 21 °C and 2 days at 30 °C. sP-selectin was not stable in plasma or whole blood. sICAM-1, sVCAM-1, CRP, and sE-selectin were stable after 5 freeze-thaw cycles.

**Conclusions:** sVCAM-1, sICAM-1, and CRP are stable in plasma or whole blood at 4 and 21 °C for at least 3 days and sE-selectin for 2 days. sP-selectin is not stable and therefore requires immediate assay.

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Multicenter studies that measure the cardiovascular risk markers soluble intracellular adhesion molecules-1 (sICAM-1), soluble vascular cell adhesion molecules-1 (sVCAM-1), soluble (s)E-selectin, sP-selectin, and C-reactive protein (CRP) in serum or plasma require validated procedures for processing, storage, and transport of sam-

ples. Previous studies have not systematically examined effects on these markers of different storage temperatures, duration of storage, freeze-thaw cycles, or delays in separating plasma from whole blood (1–5). Venous blood samples were obtained from 10 nonsmoking healthy staff members (7 male, 3 female, age 24–58 years) who had given informed consent. Clinical research nurses collected the samples into 3 7-mL K3 EDTA glass tubes and 12 2-mL K3 EDTA glass tubes (Becton Dickinson) per person. Samples were randomized into 3 groups. Group 1 samples [six 0.5-mL samples from 5 volunteers (4 males) (see Fig. 1 in the Data Supplement that accompanies this article at <http://www.clinchem.org/content/vol53/issue10>)] were centrifuged immediately, stored at –80 °C, and then subjected to 0–5 room temperature freeze-thaw cycles. Group 2 samples [1 2.5-mL samples from each volunteer (Supplementary Data Fig. 2)] were centrifuged immediately, and the plasma was then divided into 13 aliquots. One aliquot was frozen immediately, and the others were maintained at 4, 21, or 30 °C for 1, 2, 3, or 5 days and then frozen at –80 °C until assayed. Group 3 samples [1 3-mL sample from each volunteer (Supplementary Data Fig. 2)] were split into 13 aliquots, 1 aliquot was centrifuged immediately and the plasma stored at –80 °C, and the remaining 12 aliquots were stored as whole blood under the same conditions as group 2 before centrifugation and freezing of the plasma at –80 °C. Frozen aliquots were packed in dry ice and sent to the VU University Medical Centre, The Netherlands, where samples were thawed, inverted, and vortex mixed before analysis.

All aliquots were assayed in duplicate at room temperature in batches of 39 samples in up to 2 runs per sampling method for each analyte, including the freeze-thaw aliquots, using semiautomated ELISA methods for sVCAM-1, sICAM-1, and high-sensitivity CRP (all obtained from Diaclone) on the CODA automated EIA reader (Bio-Rad) and manual ELISA methods for sE-selectin (Diaclone) and sP-selectin (R&D Systems) (6).

Baseline values used for each analyte were those obtained from assay of plasma from samples that had been separated immediately. The mean percentage change from baseline was calculated for each time point, temperature, and freeze-thaw cycle using the Statistical Package for the Social Sciences (v12.2, SPSS). Analyte values that differed by >10% from baseline and were statistically significant ( $P < 0.05$ ) were considered to indicate unacceptable stability for given storage/handling conditions (7). We used generalized estimating equations to assess correlations in the data and compared the different storage time and temperature values to baseline values for each sample (8).  $P$  values reported are those from generalized estimating equation models obtained using the statistical package STATA (Intercooled STATA 8.2 for Windows).

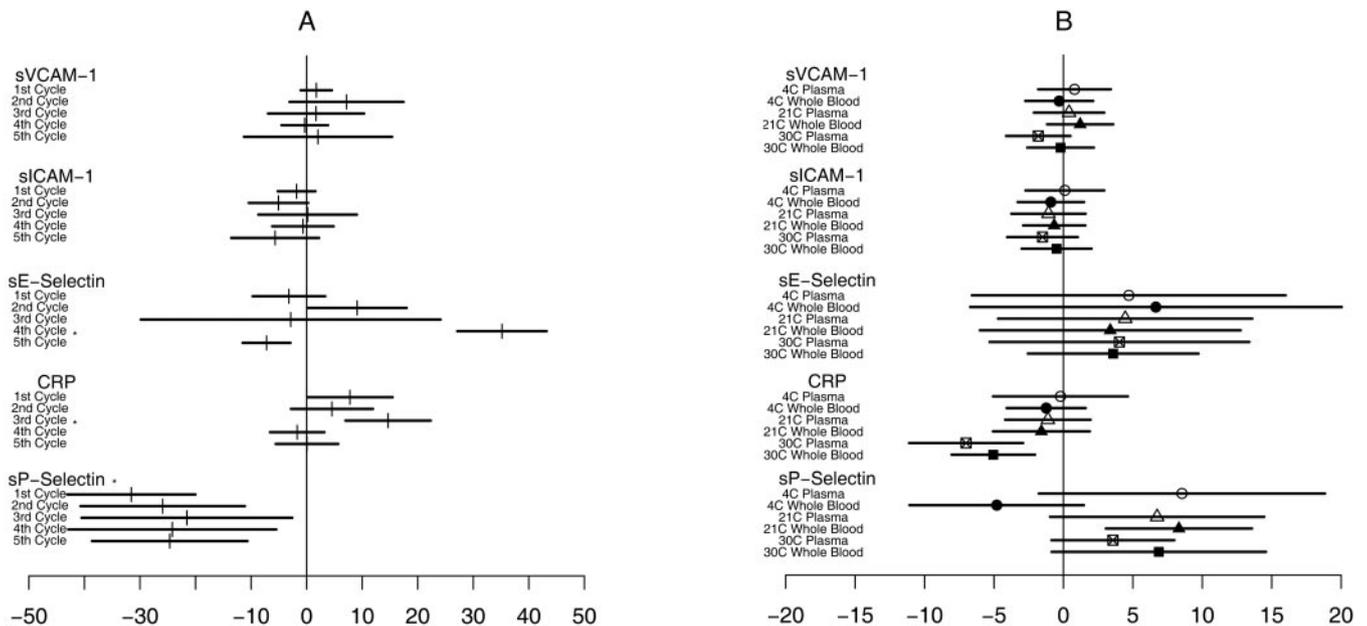


Fig. 1. (A), mean (95% CI) percentage change from baseline for each analyte after 1–5 freeze-thaw cycles.

Values that differ significantly from baseline ( $P < 0.05$ ) are denoted by an asterisk. (B), mean (95% CI) percentage day-to-day change for days 0–1, 1–2, 2–3, and 3–5 for each analyte, calculated using robust variance estimates and adjusting for plasma or whole blood samples showing variation after storage at prespecified temperatures (○ = 4 °C plasma; ● = 4 °C whole blood; △ = 21 °C plasma; ▲ = 21 °C whole blood; □ = 30 °C plasma; ■ = 30 °C whole blood).

Mean (range) baseline values for sVCAM-1 were 873 (715–1283)  $\mu\text{g/L}$ , sICAM-1 491 (350–864)  $\mu\text{g/L}$ , sE-selectin 41 (31–75)  $\mu\text{g/L}$ , high-sensitivity CRP 4 (0.17–12.15)  $\text{mg/L}$ , and sP-selectin 29 (11.5–40.1)  $\mu\text{g/L}$ . These values were all within published reference intervals (9–12) after removal of probable outliers (values  $>4$  SD from the mean) (13). Interassay CVs ranged from 1.3% to 2.4% at 660 to 703  $\mu\text{g/L}$  for sVCAM-1, 3.6% to 5.6% at 348 to 485  $\mu\text{g/L}$  for sICAM-1, 9.9% to 10.0% at 14.2 to 18.3  $\mu\text{g/L}$  for sE-selectin, 3.0% to 6.1% at 0.78 to 1.59  $\text{mg/L}$  for CRP, and 9.0% to 9.4% at 30.2 and 38.2  $\mu\text{g/L}$  for sP-selectin. Intraassay CVs were 4.4% for sVCAM-1, 4.0% for sICAM-1, 4.0% for sE-selectin, 3.9% for CRP, and 2.7% for sP-selectin. VCAM-1 and sICAM-1 were stable up to 5 days under all storage conditions in plasma and whole blood, and after 5 freeze-thaw cycles (Table 1), although whole blood gave more reproducible results than plasma for sICAM-1. sE-selectin was stable for up to 2 days at 4 °C in plasma and whole blood and for 5 freeze-thaw cycles. CRP was stable for 5 days at 4 and 21 °C in plasma

and whole blood and for 5 freeze-thaw cycles. sP-selectin was unstable under all storage conditions and thus requires immediate processing (Fig. 1 and Table 1) (also see Supplemental Data Table 1 and Supplemental Data Table 2 for all results).

This study is the 1st to assess the effects of sample storage for up to 5 days at different temperatures and exposure to repeat freeze-thaw cycles on stability of soluble adhesion molecule and selectin measurements in whole blood and plasma. Previous studies of CRP stability (1, 2, 5, 8, 14–16) did not consider repeated freeze-thaw cycles or higher storage temperatures beyond 3 days in plasma vs whole blood, and investigated only the effects of storage beyond 5 days at 4 or 21 °C. Our sample size was too small to assess concentration-dependent effects, which should be assessed in a similar manner in future studies.

Assays were conducted at an ambient temperature of 21 °C in single batches by 2 laboratory technicians. To improve precision and run-to-run variability, 1 technician used semiautomated analysis methods on 3 of the markers

**Table 1. Number days for which analyte values did not differ significantly ( $P \geq 0.05$ ) from baseline, with  $<10\%$  variation after storage as plasma or whole blood at 4 °C, 21 °C, or 30 °C or after a number of freeze-thaw cycles.**

	Temperature	sVCAM-1	sICAM-1	sE-selectin	CRP	sP-selectin
Plasma (days)	4 °C	5	5	2	5	0
	21 °C	5	5	5	5	0
	30 °C	5	5	2	1	0
Whole blood (days)	4 °C	5	5	2	5	0
	21 °C	5	5	5	5	0
	30 °C	5	5	2	2	0
Freeze-thaw cycles (n)	–80 °C	5	5	5	5	0

(sVCAM, sICAM, CRP), and manual methods were used on the other assayed selectins. A few measurements do not fit any discernable pattern, notably plasma sVCAM-1 on day 3 at 4 and 21 °C; plasma sE-selectin on day 1 at 4 °C and day 3 at all temperatures, whole blood sE-selectin at 4 °C on day 1 and after freeze-thaw cycle 4; and plasma CRP on day 1 at 4 °C and after freeze-thaw cycle 3. These aberrant values were likely analytic artifacts, because removal of values >4 SDs from the mean or adjustment for run-to-run variability did not change the results qualitatively. The large CRP difference observed after freeze-thaw cycle 3 but not after cycles 4 or 5 suggests assay error or possibly CRP release from LDL and complement factors. It has been demonstrated that a portion of systemic CRP is bound to cholesterol in modified LDL particles (17) and to different complement factors (18). Interaction of CRP with cholesterol and complement factors might hamper the detection of CRP in the assay used. No data were available for sE-selectin, but it is possible that some sE-selectin is bound to different circulating leukocyte types or microvesicles derived from endothelial cells. Freeze-thaw cycles might release these bound forms of sE-selectin, leading to an increase in detectable sE-selectin.

Possible effects of residual platelets in the plasma on marker values obtained are not addressed in our study. sVCAM-1, sICAM-1, and sE-selectin, however, are derived mainly from endothelial cells and not from platelets. Although the presence of platelets in EDTA plasma might contribute to the absolute amounts of these markers, it does not influence their stability. In contrast, sP-selectin is mainly derived from platelets, a characteristic that may explain the variance observed in sP-selectin at different time and temperature points.

Our results show that sP-selectin is unstable under all storage conditions and requires immediate assay. The other cardiovascular risk markers evaluated were stable when stored in whole blood samples for several days at room temperature. sVCAM-1, sICAM-1, and CRP were stable at 4 and 21 °C in plasma or whole blood for 5 days and sE-selectin for 2 days. These findings have important implications for clinical studies measuring these markers, reducing the need for immediate transfer of samples to a laboratory for processing and analysis.

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**Background:** The blood of cancer patients is known to contain fragments of RNA released from the tumor. The

application of genomic profiling techniques to plasma RNA may allow the unbiased selection of cancer markers in the blood, but the informative value of genomic profiling of plasma RNA is currently unknown.

**Methods:** We used cDNA microarray hybridization to perform genomic profiling of plasma RNA from colorectal cancer (CRC) patients and from healthy donors. From a list of 40 genes differentially upregulated in cancer patients, we randomly selected 4 genes for further characterization. These 4 markers were analyzed by quantitative reverse-transcription PCR in a wide set of samples including paired samples from the same CRC patients before and after surgical resection of the tumor.

**Results:** Three of the selected markers—*EPAS1*, *UBE2D3*, and *KIAA0101*—were confirmed by PCR to be significantly increased in cancer compared to healthy donors. Importantly, 2 of the markers, *EPAS1* and *UBE2D3*, showed a significant decrease after surgery, returning to the levels of healthy donors. Finally, supervised class prediction using these 3 markers correctly (77%) assigned presurgery samples to the CRC group and assigned postsurgery samples from the same patients to the healthy group.

**Conclusions:** Our findings demonstrate the usefulness of gene expression profiling of circulating plasma RNA to find cancer markers of potential clinical value.

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The blood of cancer patients contains higher concentrations of DNA than does the blood of healthy individuals (1). The development of PCR amplification techniques has allowed the analysis of this circulating DNA, and a large body of evidence has demonstrated that the plasma DNA from cancer patients presents features of the cancer DNA, suggesting that it derives from tumor cells (2–5). More recently, several groups have reported the extraction of RNA from the plasma of cancer patients and its subsequent analysis by reverse transcription (RT)-PCR (6–10). The potential use of plasma RNA for the analysis of cancer is highly attractive for several reasons: it requires a minimally invasive method (collection of a small amount of blood); it can be obtained at any time and in a repetitive fashion, allowing the analysis of disease progression and treatment response; and its simplicity is amenable for use in asymptomatic populations at risk. The analysis of plasma RNA has been restricted to a few markers assumed to be abundant and specifically associated with particular cancer types, for example, hemoglobin for breast cancer and tyrosinase for melanoma (7, 10). Further progress toward the clinical use of plasma RNA requires the unbiased identification of markers. Genome-wide profiling of plasma RNA is an obvious approach but has technical drawbacks that could prevent its application, such as the low abundance and lack of integrity of plasma RNA (11).

For this reason we evaluated the feasibility of a genomic approach to studying plasma RNA. We measured by

cDNA microarray hybridization the relative abundance of the different RNA species in the plasma of colorectal cancer (CRC) patients ( $n = 12$ ) and healthy donors ( $n = 8$ ). All the patients and healthy donors along this study gave their informed consent following the rules of the Research Ethics Board of Hospital Universitario Puerta de Hierro. Each sample was competitively hybridized against a common reference formed by a pool of blood samples from 26 healthy donors different from those hybridized as individual healthy samples. Differential gene expression analysis between CRC and healthy donors identified a total of 87 genes, including 40 that were differentially upregulated in the cancer group (see Supplementary Fig. 1A and Supplementary Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at <http://www.clinchem.org/content/vol53/issue10>). Comparison with previous gene expression analysis of CRC that used tissue from the tumor as the source of RNA revealed that 4 of our differentially upregulated genes, *PSAM3*, *RANBP1*, *GCLC*, and *KIAA0101*, had been previously identified in 2 different studies as upregulated in CRC (12, 13).

We then performed quantitative RT-PCR (Q-RT-PCR) on the same samples to analyze expression for a subset of 4 randomly selected genes, *KIAA0101*, *UBE2D3*, *EPAS1*, and *DDX46*, from the list of 40 differentially upregulated genes. Two of them were validated by PCR as significantly upregulated in the CRC samples (*KIAA0101* and *UBE2D3*; both with Kruskal–Wallis (KW)  $P$  value  $< 0.05$ ), one showed clearly increased expression although with lower statistical significance (*EPAS1*; KW  $P$  value = 0.098), and the last one could not be validated (*DDX46*;  $P$  value = 0.96) (Table 1 and Supplementary Fig. 1B in the online Data Supplement).

One of these 4 genes, *KIAA0101*, also known as *p15PAF*, has been previously identified as a commonly overexpressed gene in a variety of solid tumors by 2 independent groups who used large-scale metaanalysis of cancer DNA microarray data (14, 15). *EPAS1* encodes hypoxia-inducible factor 2- $\alpha$  (HIF2 $\alpha$ ), an important angiogenic factor whose high expression in CRC has been shown to play an important role in tumor progression and to possess prognostic value (16). *UBE2D3*, another of our selected markers, encodes a ubiquitin-conjugating enzyme, also known as UBCH5C, involved in the regulated degradation of important cellular factors such as the tumor suppressor p53 and the NF $\kappa$ B regulator, I $\kappa$ B $\alpha$  (17, 18). Finally, *DDX46* encodes a member of the DEAD box protein family that has putative helicase activity and is involved in pre-mRNA splicing as part of the 17S U2 small nuclear ribonucleoprotein complex.

To test the consistency of the detection of these genes in the blood of CRC patients, we analyzed their expression by Q-RT-PCR on a set of 29 new CRC plasma samples and 36 healthy donor samples, different from the ones that were part of the microarray study. With this external set we verified the increased expression of 2 of the markers, although only 1 of them (*EPAS1*, KW  $P$  value  $< 0.05$ ) was significantly higher in CRC patients than in healthy

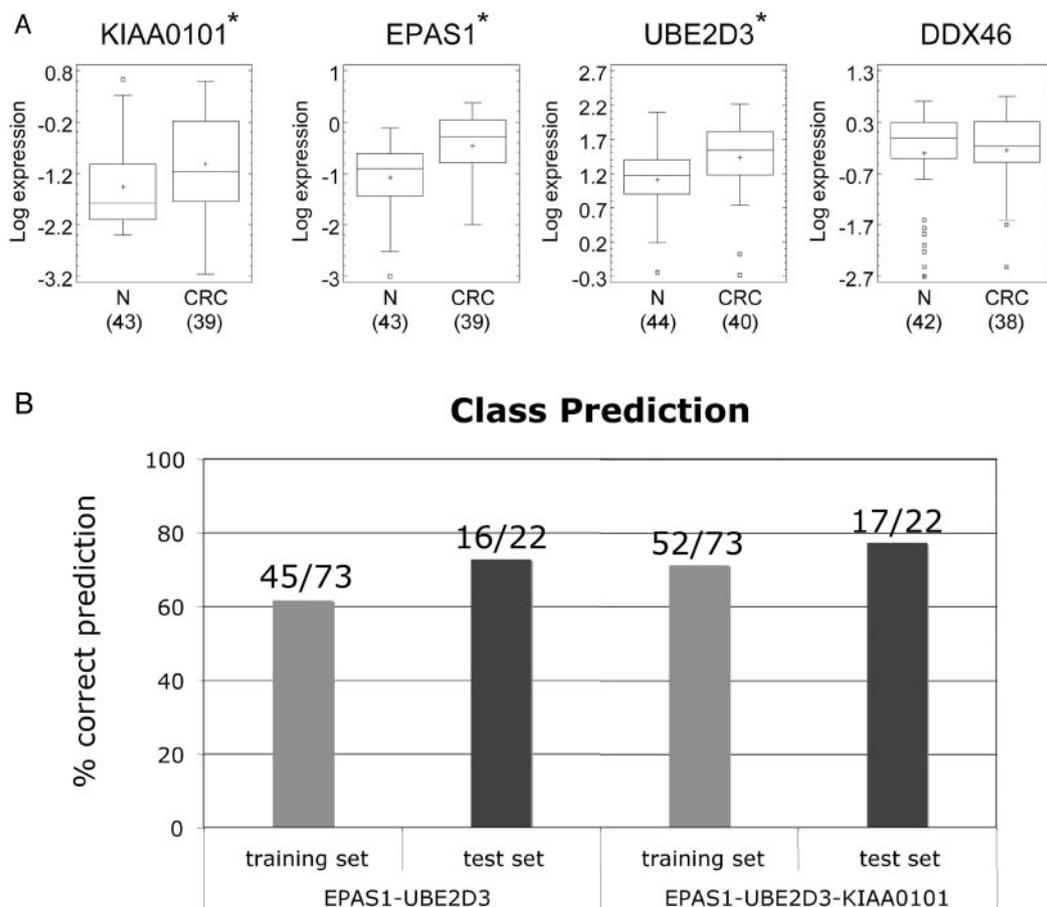


Fig. 1. Q-RT-PCR analysis of CRC markers in plasma.

(A), analysis of the mRNA concentrations of the selected markers in plasma of the complete series (internal plus external, see Table 1) of normal (N) and CRC patients (CRC) samples used throughout the study (the number of samples in each case is shown below the class label). Genes for which a statistically significant difference ( $P < 0.05$ ) was shown by KW test are marked with (\*). (B), class prediction using Q-RT-PCR expression data of our markers. A SVM algorithm with lineal kernel was applied to construct a model for class prediction using Q-RT-PCR expression data for *UBE2D3*, *EPAS1*, and *KIAA0101*, or *UBE2D3* and *EPAS1* together, for all the samples used in this study except for the pre- and postsurgery samples (□: training set). The models generated were used to classify the pre- and postsurgery samples (■: test set). The graph shows the percentage of correctly classified samples in each case. Correctly classified and total number of samples are shown on top of each column.

donors. The other marker (*UBE2D3*, KW  $P = 0.09$ ), although its mean value was increased, had lower statistical significance (Table 1 and Supplementary Fig. 2A in the online Data Supplement). Finally, the overall analysis of the above markers by Q-RT-PCR across all the samples used in this study, i.e., the internal and the external sets together, showed a clear and statistically significant (KW  $P < 0.05$ ) increase of *KIAA0101*, *UBE2D3*, and *EPAS1* in the plasma of CRC patients compared to healthy individuals (Fig. 1A).

To test the power of the identified markers in enabling differentiation of the tumor from the healthy condition, we measured the levels of these markers in the plasma of CRC patients ( $n = 11$ ), from whom it was possible to obtain blood samples after surgical removal of the tumor. Importantly, *EPAS1* and *UBE2D3* were significantly decreased in the postsurgery samples compared to the presurgery samples from the same patients (Table 1 and Supplementary Fig. 2B in the online Data Supplement).

To further explore the discriminating power of the markers identified in this study, we applied a supervised learning algorithm to the Q-RT-PCR dataset, excluding the pre- and postsurgery data and using the resulting dataset as the training set. Support vector machine (SVM) analysis with leave-one-out cross-validation of this training set using the 3 validated genes *UBE2D3*, *EPAS1*, and *KIAA0101*, showed that they enabled classification of up to 71% of the training samples correctly (52 of 73 samples) (see Fig. 1B). Use of only 2 markers, *UBE2D3* and *EPAS1*, did not improve scores obtained with the 3 genes together. Using the model generated by SVM with the 3 markers, we performed class prediction on a test set composed of the Q-RT-PCR data derived from the pre- and postsurgery group. In this way, we classified 77% of the samples correctly (17 of 22 samples), i.e., presurgery samples were classified as CRC and postsurgery samples were classified as normal (Fig. 1B). The misidentified samples were 3 presurgery samples that were wrongly

**Table 1. Summary of expression values and statistical analysis for the selected CRC markers across the internal, external, and pre- and postsurgery groups (n = number of samples in each case).**

	Gene symbol			
	KIAA0101	EPAS1	UBE2D3	DDX46
Fold change microarray	4.5	5.5	4.1	4.1
Internal				
Fold change Q-PCR	6.6	6.4	5.1	1.3
Normal (n) CRC (n)	(8) (11)	(8) (11)	(8) (11)	(8) (11)
KW <i>P</i> -value	0.01	0.09	0.001	0.96
External				
Fold change Q-PCR	1.5	2.8	1.6	1.1
Normal (n) CRC (n)	(35) (28)	(35) (28)	(36) (29)	(34) (27)
KW <i>P</i> -value	0.14	0.0002	0.09	0.94
Pre/Post				
Fold change Q-PCR	0.5	0.1	0.6	1.2
Presurgery (n)	(11) (11)	(11) (11)	(11) (11)	(11) (11)
postsurgery (n)				
KW <i>P</i> -value	0.72	0.0002	0.007	0.39

classified as normal and 2 postsurgery samples assigned to the CRC group.

Simultaneous monitoring of the expression of numerous genes by DNA microarrays provides a powerful tool in medical research, but the widespread clinical application of DNA microarrays is hindered by the need for sample collection directly from the tumors. Analysis of circulating RNA in the plasma circumvents this limitation, making sample collection easy and reproducible, and allowing for reiterative extractions during treatment response. This proof-of-concept study demonstrates the feasibility of such an approach. Our results provide an example of the power of plasma RNA analysis to differentiate tumor from the healthy condition in a clinical setting. We observed that some of our markers, present at high concentrations in CRC, returned to normal after surgical removal of the tumor. Furthermore, class prediction using SVM classified the presurgery samples as members of the CRC group and the postsurgery samples as part of the normal group.

On the basis of our results, large-scale gene expression profiling of a large number of samples should yield candidate markers of potential diagnostic and prognostic value.

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