Addition of Exogenous Reporter Peptides to Serum Samples before Mass Spectrometry-Based Protease Profiling Provides Advantages over Profiling of Endogenous Peptides

To the Editor:
Serum has proven a difficult matrix for mass spectrometry (MS)-based clinical proteomic profiling. Problems occur mainly as a result of preanalytical variables in sample handling and processing, causing substantial changes in MS peptide profiles (1) that can completely abolish meaningful data interpretation (2). The necessary rigorous standardization of sample collection and processing procedures is difficult to integrate into routine laboratory testing. Furthermore, no new biomarker proteins have emerged from profiling experiments of serum specimens; thus far only proteolytic fragments of high-abundance serum proteins have been reported as markers (3). Proteases shape the peptide pattern of serum specimens in a time-dependent manner (1), and disease-related proteases seem to generate a characteristic pattern of proteolytic fragments from abundant endogenous proteins (4).

We hypothesized that addition of exogenous reporter peptides (RPs) to serum will also enable characterization of protease activity in serum samples while offering a major advantage: The standardization of RP addition with respect to incubation time, temperature, and substrate concentration is quite simple and might alleviate the preanalytical difficulties of current profiling approaches.

To test this hypothesis we generated an RP mixture by tryptic digestion of a randomly chosen recombinant protein. The N-terminally 6×His-tagged fragment of the adenomatous polyposis coli protein that covers amino acids 3–312 (NP_000029) was expressed using the pQE30 vector (Qiagen) and Escherichia coli XL1-Blue cells. Protein was purified using Ni-Sepharose before carboxamidomethylation and in-solution digestion with trypsin (Promega). The resulting peptides were purified using Omix® C18 pipette tips (Varian), then organic solvent of the eluate was evaporated and peptides were reconstituted with PBS, pH 7.4 (cat. no. H15-002; PAA Laboratories), to yield the final RP mixture solution.

We mixed 30 μL of serum and 20 μL of the RP mixture and incubated the mixture at 37 °C for 2 h. In control samples, we substituted 20 μL PBS for the RP mixture. Before MS (MALDI-TOF Autoflex II, Bruker) the samples were processed using magnetic bead-based hydrophobic interaction chromatography on C18resins (Bruker). Blood was collected from 50 healthy employees of the University Hospital Mannheim during routine laboratory testing at the employee medical facility. All specimen donors gave informed consent, and the local ethics committee approved the study.

Serum was centrifuged at 20 °C for 10 min at 3000g and stored at −80 °C. To investigate preanalytical impact on MS profiles, the serum of 1 donor was divided into aliquots and stored at room temperature for 1–6 h before freezing at −80 °C. For measurements, all aliquots were thawed once and processed in parallel with or without the addition of the RPs (Fig. 1A).

The MS patterns of peptide-supplemented sera were comparable among samples from healthy individuals. Within-day and between-day reproducibility of MS-spectra were assessed as previously described (1), and the coefficient of determination ($R^2$) in any case was ≥0.93 (data not shown), confirming good reproducibility of magnetic bead-based sample processing. Incubation of RPs with serum changed the peptide profile. By comparing samples before and after incubation for 2 h at room temperature, we distinguished the following peak categories (Fig. 1B): Peaks designated category A originated from serum. Peaks designated category B were RPs of the RP mixture that were not found in the sera after addition of the RP mixture, suggesting that they either were rapidly degraded by serum proteases or were quenched owing to ion suppression. Peptides designated category C originated from the RP mixture but with varying signal intensities. Peaks designated category D appeared de novo in serum as a result of proteolytic cleavage of RP peptides by endogenous proteases.

We designated 2 different preanalytical periods, periods I and II, as outlined in Fig. 1A. As exemplified in Fig. 1B, different extensions of preanalytical period I resulted in distinct changes of MS-peptide profiles and a time-dependent decrease of the m/z 1466 category A peak (1). In contrast, category D peaks (m/z: 1364, 1741, and 1899; Fig. 1B) that appeared in enriched sera did not show differences in signal intensity despite variability in preanalytical period I, demonstrating that protease activity was unaffected over prolonged periods of sample storage.

In conclusion, these preliminary results demonstrate that addition of RP to serum specimens can alleviate preanalytical variability. The feasibility of RP addition has already been demonstrated for diagnosis of thrombotic thrombocytopenic purpura associated with altered activity of a single protease (5). A future challenge will be the composition of an optimized RP mixture for MS-based protease profiling of other conditions such as malignancies that are also associated with specific protease patterns.

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References
1. Findsen P, Sismanidis D, Riedl M, Costina V, Neumaier M. Preanalytical impact of sample...
Fig. 1. (A), workflow for analysis of preanalytical influence on MS-profiles of serum with RP addition.

One serum sample was stored at room temperature for different time periods (preanalytical time period I), and aliquots were frozen at −80 °C every hour for up to 6 h. After thawing, aliquots were further processed in parallel. RP mixture was added at 0–6 h (S0h–S6h) or PBS was added (P0h–P6h). The aliquots were then incubated for 2 h at 37 °C (RP addition time period II) before MB-HIC C18 purification and MALDI-TOF-MS. (B), preanalytical influence on MS profiles and stability of signals after RP addition. Pseudo-gel view of pure serum (upper graph), serum with added RPs (middle graph), and RP mixture (lower graph) in a magnified area of m/z 1000–2200; signal intensity is coded by grade of blackening. Examples of peaks of categories A through D (see text) are indicated by letters. The peak m/z 1364 was identified as a fragment (ECSPVPMGSFPR) of the adenomatous polyposis coli protein showing N-terminal processing of the category C peak m/z 1509 (RSGECSPVPMGSFPR_R) that is in the RP mixture. (Trypsin cleavage sites are indicated by underscore, cysteine residues showed modifications due to iodoacetamide treatment.)


Relationship between 
\(\gamma\)-Glutamyltransferase, Fasting Plasma Glucose, and Triglycerides in the General Population

To the Editor:

Recent population-based epidemiologic studies have convincingly shown that serum \(\gamma\)-glutamyltransferase (GGT) activity is associated with many cardiovascular disease risk factors and predicts new-onset type 2 diabetes, hypertension, stroke, and myocardial infarction (1, 2). We read with interest the recent article by Lim et al. (3) on the possible interaction between GGT and obesity and its association with the risk of prevalent type 2 diabetes, findings indicating that obesity itself may not be a sufficient risk factor for diabetes when GGT concentrations approach the lower limit of the reference interval. The clinical implications of this conclusion are noteworthy because overweight-obese people with GGT concentrations at the lower limit of the reference interval (e.g., <20 U/L) would no longer be considered at high risk of developing diabetes. To further investigate the relationships among GGT, diabetes, and other major biochemical components of the metabolic syndrome in the general population, we analyzed whether serum GGT concentrations predict prevalent diabetes and hypertriglyceridemia, and whether there is an interaction between GGT and hypertriglyceridemia that affects the risk of prevalent diabetes.

We performed a retrospective analysis on the database of the laboratory information system of the clinical chemistry laboratory at the Verrona University Hospital to retrieve test results for serum GGT, fasting plasma glucose (FPG), and triglyceride, which had been performed on the whole cohort of outpatients consecutively referred by general practitioners for routine blood testing in the preceding 9 months (August 2006–April 2007). Venous blood from outpatients was routinely collected in the morning from fasting individuals, and FPG, triglycerides, and GGT were assayed by enzymatic procedures on a Roche/Hitachi Modular System (Roche Diagnostics GmbH). We assessed the significance of differences and frequency distributions of values with the Kruskal-Wallis test and the \(\chi^2\) test (for categorical variables), respectively. Multivariable logistic regression analysis was used to examine the interaction relationship with diabetes, as the dependent variable, predicted from triglycerides (subdivided in tertiles: \(<0.95, 0.95–1.47, \) and \(>1.47\) mmol/L) within 3 categories of GGT (\(<20, 20–39.9, \) and \(>40\) U/L). Adjusting variables were age and sex. Statistical analyses were performed using the statistical package SPSS-version 12.

Cumulative results for GGT, FPG, and triglycerides were retrieved for 7267 outpatients >35 years old during a 9-month period. As shown in Table 1, the concentrations of FPG and triglycerides markedly increased among the GGT categories. Similarly, the frequencies of Lim et al. (3) that GGT concentrations at the lower limit of the reference interval (e.g., <20 U/L) would no longer be considered at high risk of developing diabetes. To further investigate the relationships among GGT, diabetes, and other major biochemical components of the metabolic syndrome in the general population, we analyzed whether serum GGT concentrations predict prevalent diabetes and hypertriglyceridemia, and whether there is an interaction between GGT and hypertriglyceridemia that affects the risk of prevalent diabetes.

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Cumulative results for GGT, FPG, and triglycerides were retrieved for 7267 outpatients >35 years old during a 9-month period. As shown in Table 1, the concentrations of FPG and triglycerides markedly increased among the GGT categories. Similarly, the frequency of those with FPG \(\geq 7.0\) mmol/L, a cutpoint suggestive for diagnosing diabetes according to the American Diabetes Association guidelines (4), and of those with hypertriglyceridemia (\(>1.7\) mmol/L by the Third Adult Treatment Panel criteria) increased steadily across the spectrum of GGT thresholds from 16% to 31% for FPG and from 14% to 39% for triglycerides, respectively. These results remained unchanged after we adjusted for sex and age. Interestingly, as also shown in Table 1, the age-, and sex-adjusted frequencies of diabetes significantly increased across the tertiles of triglycerides within all 3 GGT categories, thus excluding the existence of a significant interaction between GGT and triglycerides on the risk for prevalent diabetes.

Increased concentrations of GGT, an enzyme associated with liver damage (2), are conventionally interpreted as a marker of alcohol consumption. However, as previously reported, alcohol consumption could not entirely explain the association between increased GGT concentrations and type 2 diabetes, because this association was observed even after adjustment for daily alcohol intake and existed among nondrinkers (1, 2). Moreover, it has been shown that increased GGT concentrations are associated with lipid abnormalities independently of obesity (5). In the present study, we failed to find a significant interaction between GGT and triglycerides in predicting prevalent diabetes, suggesting that the association between triglycerides and diabetes might be only marginally influenced by serum GGT concentrations. Obviously, we must be cautious in making any causal inference because of the cross-sectional nature of our study, but such findings would not be unexpected because of the intertwined and complex biological relationships linking diabetes and hypertriglyceridemia. Nevertheless, our results do not exclude the possibility that increased GGT, as a marker of fatty liver, plays an important role in the development of type 2 diabetes (1, 2).

Overall, we agree with the suggestions of Lim et al. (3) that GGT
measurement may be useful in clinical settings for detecting high-risk subpopulations of type 2 diabetes and/or hypertriglyceridemia. Such individuals might benefit from a more intensive therapeutic approach to decrease their global cardiovascular risk, regardless of potential unmeasured effects of lifestyle or obesity. Conceivably, the significant association of serum GGT concentrations with FPG and triglycerides, observed in our investigation, may be biologically explained by some underlying mechanisms such as hepatic steatosis, insulin resistance, and increased oxidative stress.

References


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Effect of Serum Gamma-Glutamyltransferase and Obesity on the Risk of Dyslipidemia and Poor Glycemic Control in Type 2 Diabetic Patients: Cross-Sectional Findings from the Verona Diabetes Study

To the Editor:

We read with interest the article by Lim et al. (1) regarding a strong interaction between serum gamma-glutamyltransferase (GGT) activity and body mass index (BMI) and their effect on the risk of prevalent diabetes. The authors found that BMI is associated with prevalent diabetes only among individuals with high-normal GGT, suggesting that GGT determination can be useful in clinical settings for identifying individuals at high risk for diabetes.

Given the scientific and clinical importance of an interaction between obesity and GGT in predicting diabetes, we investigated possible interactions between BMI and GGT in predicting poor glycemic control and common comorbidities of diabetes. Therefore, we assessed whether the association of BMI with hypertension, dyslipidemia, and poor glyce-
mic control differed according to serum GGT activities in a type 2 diabetic population.

Study participants were enrolled in the Verona Diabetes Study, a pro-
spective observational study designed primarily to evaluate associa-
tions between type 2 diabetes and incidence of cardiovascular compli-
cations. In this analysis, we included 2929 type 2 diabetic outpa-
tients [56% males; mean (SD) age 68 (10) years] who regularly attended
our clinic and who had complete data for analysis. Fasting serum GGT
and lipid values were determined by standard laboratory procedures
(Roche Diagnostics). Hemoglobin A1c was measured with an HPLC
analyzer (Bio-Rad Diamat); the up-
standard laboratory procedures
value for interaction
association of BMI with each outcome measure was significant, the
assonations varied remarkably by
BMI categories, kg/m²
<25), %25–29.9, %30–34.9, and
and hypertension did not substan-
tially change across GGT categories.
For example, within the lowest GGT
quartile, BMI was not associated with dyslipidemia or worse glycemic
control, in contrast to the highest
GGT quartile, wherein the prevalence
rates were 60%–78.5% for dys-
lipidemia and 50.4%–72.4% for glyce-
mic control, respectively. Notably,
these results were observed after ad-
justment for sex, age, and diabetes
duration and treatment.

Our findings suggest that in people
with type 2 diabetes, BMI is associated
with worse glycemic control and dys-
lipidemia only among those with
high-normal GGT, but not in those
with low-normal GGT. These findings,
although only correlative in nature,
complement the cross-sectional obser-
vations by Lim et al. (1), suggesting
that the association of BMI with type 2
diabetes and its related metabolic dis-
orders remarkably varied with serum
GGT activity, and that obesity itself
may not be a sufficient risk factor for
developing diabetes, dyslipidemia, or
worse glycemic control.

The associations between GGT ac-
tivity and diabetes and its related
comorbidities may possibly be ex-
plained by some underlying biologi-
cal mechanisms such as enhanced
oxidative stress, insulin resistance,
and fatty liver.

Markers of liver fat such as serum
GGT activity have been shown in pro-
spective studies to predict type 2 dia-
betes, insulin resistance, dyslipidemia,
and cardiovascular disease indepen-
dent of obesity (4). Moreover, obesity
appears to be unnecessary to the oc-
currence of insulin resistance in hu-
man, because severe insulin resis-
tance also characterizes patients
lacking subcutaneous fat, such as
those with lipodystrophy. A close lin-
ear relationship exists between liver fat
content and direct measures of hepatic
insulin resistance (4). The fatty liver
thus might help to explain why some
but not all obese individuals are insu-
lin resistant and why even lean indi-
viduals may be insulin resistant and
thereby at risk of developing type 2
diabetes and its related metabolic dis-

### Table 1. Prevalence rates of hypertension, atherogenic dyslipidemia, and poor glycemic control by category of BMI after stratification by quartile of serum GGT activity in type 2 diabetic population (n = 2929).

<table>
<thead>
<tr>
<th>GGT quartiles, U/L</th>
<th>Hypertension</th>
<th>Dyslipidemia</th>
<th>Poor glycemic control</th>
<th>Hypertension</th>
<th>Dyslipidemia</th>
<th>Poor glycemic control</th>
<th>P values for trend adjusted for age, gender, diabetes duration, and treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;16 (n = 703)</td>
<td>68.9</td>
<td>57</td>
<td>61.8</td>
<td>72.6</td>
<td>60</td>
<td>58.3</td>
<td>&lt;0.001, 0.38, 0.87, &lt;0.005, 0.02, 0.63, &lt;0.001, 0.40, &lt;0.001, 0.045, &lt;0.001</td>
</tr>
<tr>
<td>16–25 (n = 792)</td>
<td>79.8</td>
<td>61.2</td>
<td>65.7</td>
<td>77.3</td>
<td>64.6</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>26–43 (n = 715)</td>
<td>86.3</td>
<td>61.9</td>
<td>55.7</td>
<td>79.6</td>
<td>67.9</td>
<td>60.2</td>
<td></td>
</tr>
<tr>
<td>≥44 (n = 719)</td>
<td>87</td>
<td>66.9</td>
<td>59.7</td>
<td>85.5</td>
<td>71.5</td>
<td>67.1</td>
<td></td>
</tr>
</tbody>
</table>
orders. To further complicate the intertwined biological links between obesity and diabetes, it has been shown that obesity does not increase the prevalence of diabetes among those with undetectable serum concentrations of persistent organic pollutants (5).

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To the Editor:
We read with interest the 2 letters addressing our recent article on the interactive effects of obesity and serum γ-glutamyltransferase (GGT) on the risk of type 2 diabetes. We reported that obesity, a well-established risk factor of type 2 diabetes, was not associated with type 2 diabetes among the approximately 50% of participants with low-normal serum GGT, whereas obesity was associated with risk of diabetes in the other half of participants who had high-normal serum GGT.

Targher et al.’s cross-sectional findings among type 2 diabetes patients, reported above, appear to support our findings, despite a study purpose and population that differed from ours. Targher et al. reported that associations between obesity and the risk of dyslipidemia and poor glycemic control were observed only among type 2 diabetic patients with high-normal and abnormal GGT, although the association between obesity and hypertension was seen at all GGT concentrations. Lippi et al., in a methodologically more limited study restricted to hospital laboratory values, failed to find any difference in the relation of hypertriglyceridemia to increased fasting plasma glucose across concentrations of serum GGT, although serum GGT itself was associated with both type 2 diabetes and hypertriglyceridemia. No measure of adiposity was available. Because the interaction of serum GGT with obesity may have a specific meaning in terms of cause and/or pathophysiology of type 2 diabetes (see below), Lippi et al.’s study of the interaction between serum GGT and hypertriglyceridemia in predicting concurrent type 2 diabetes may not be pertinent to our findings.

The authors of both letters interpreted serum GGT largely as a marker of fatty liver or oxidative stress. Although both these correlates of serum GGT could be involved, our current thinking is that a more important consideration may be the role of serum GGT as a cumulative biomarker of exposure to xenobiotics. Cellular GGT is necessary to metabolize certain xenobiotics (1). We have recently reported dose-response relations of serum or urinary concentrations of environmental pollutants [such as lead or cadmium (2) or persistent organic pollutants (POPs) (3)] with serum GGT within its reference interval in the general population. Among various xenobiots, in relation to type 2 diabetes, POPs stored in adipose tissues may be the most relevant. Parallel to the interaction between obesity and serum GGT on the risk of type 2 diabetes and insulin resistance, there were interactions between obesity and serum concentrations of POPs associated with the risk of type 2 diabetes (4). Thus, we hypothesized that the risk of type 2 diabetes would be increased among obese persons with substantial amounts of POPs in their adipose tissue, which are reflected as high-normal serum GGT, but that the risk of type 2 diabetes might not be increased among obese persons without substantial amounts of POPs in their adipose tissue, reflected as low-normal serum GGT.

Broadly speaking, this interpretation may not contradict the prevailing concept of serum GGT as a marker of fatty liver, because POP exposure may increase the risk of fatty liver as well as type 2 diabetes; in fact, serum concentrations of POPs were also associated with the prevalence of metabolic syndrome in the US general population (5). Etiologically, however, it may be important to determine whether serum GGT predicts clinical outcomes as an exposure marker of xenobiotics such as POPs or as a marker of fatty liver itself. In fact, components related to metabolic syndrome, such as dyslipidemia and/or fatty liver, may be regarded as outcomes consequent to POP exposure (5), similar to type 2 diabetes. In this sense, it is questionable whether the interaction between serum GGT and triglycerides on the risk of type 2 diabetes, which was tested in Lippi et al.’s laboratory data set, reflects the same biological mechanism as does the interaction of serum GGT with obesity. However, in Targher et al.’s work we do not rule out serum GGT as, in part, a marker for the existence of fatty liver because the associations of obesity

98493   85557   70891   56225   41559   26894   12228   3509   1869
with dyslipidemia and poor glycemic control were observed among individuals with substantially increased serum GGT (≥44 U/L).

In terms of clinical application, whatever the mechanism, the findings of Targher et al. and our findings support use of serum GGT to detect a primary target population for early intervention for prevention of type 2 diabetes and its complications.

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References
corticoids UFF + UFE as an outcome. In both models, the only significant associations observable for men were between GC3 and UFF ($\beta$, 3.7; $P < 0.01$) and GC3 and UFF + UFE ($\beta$, 10.3; $P < 0.01$). In women, in addition to a priori adjusted GC3, nitrogen also showed an association with UFF, resulting in a total explained variation of 47% (Table 1) for UFF alone. However, with UFF + UFE as the outcome, total $R^2$ in women increased to 0.72 and—in addition to urinary nitrogen—plasma leptin also explained a significant portion of variation of potential fGcA after adjustment for glucocorticoid secretion (GC3; Table 1). In line with the known stimulating effect of increased 5a-reductase activity on cortisol clearance, a trend ($P = 0.057$) for a negative association of this enzyme’s activity index (5a-THF/THF) with UFF + UFE was seen (Table 1). Accordingly, metabolic and nutritional influences on fGcA (assessed in 24-h urine samples) can be unraveled if the influence of the adrenocortical secretory activity is taken into account (e.g., as GC3).

With both UFF + UFE and UFF (the conventional measure for fGcA), increases in the protein intake-related postmeal plasma cortisol, as reported in the literature, are mirrored by the significant positive $\beta$ values for 24-h nitrogen excretion rates. However, the frequently reported interaction of leptin with the hypothalamic-pituitary-adrenal axis and the 5a-reductase influence on cortisol clearance remain masked if only UFF is analyzed. A major limitation of our study is that no individuals with abnormal glucocorticoid values were examined. Although recent findings (2, 4) and the present data suggest that UFE may be a useful complementary analyte to UFF for a more meaningful assessment of fGcA, further studies on healthy individuals and hypercortisolemic patients are required before manufacturers can be encouraged to develop adequate multiple glucocorticoid metabolite immunoassays for clinical use.

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References

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To the Editor:
Undetectable serum thyroglobulin due to negative interference of heterophile antibodies in relapsing thyroid carcinoma

To the Editor:

Undetectable serum thyroglobulin (Tg) after thyroid-stimulating hormone (TSH) stimulation is considered the most reliable marker of cure in patients with differentiated thyroid carcinoma (DTC). Interference by Tg antibodies (TgAb) and heterophile antibodies (HAb) may lead to false decreases and increases in Tg concentrations, respectively (1, 2).

A 32-year-old woman with enlarged neck lymph nodes was referred to our center. She had undergone total thyroidectomy and radioiodine treatment 4.2 years before for pT1N1x papillary thyroid carcinoma (PTC). Six months later a neck ultrasound and rhTSH-stimulated Tg assay were negative (stimulated Tg <0.9 $\mu$g/L). Subsequently both clinical examination and Tg assay under thyroxine were performed every 12 months, with negative results.

We performed neck ultrasound, which revealed 2 round hypoechoic partially colliquated lymph nodes in the right neck (III level). A fine-needle aspiration biopsy (FNAB) was performed, and the needle-washing fluid was analyzed both by cytological examination and Tg assay (Tg-FNAB). Cytology specimens showed PTC recurrence, and FNAB-Tg was 950.70 $\mu$g/L. The serum Tg was undetectable (<0.9 $\mu$g/L) with negative

Table 1. Predictors of UFF and potential bioactive free glucocorticoids (UFF + UFE) in healthy women (n = 15).a

<table>
<thead>
<tr>
<th>Models</th>
<th>Predictors</th>
<th>$\beta$</th>
<th>$P$</th>
<th>$R^2$</th>
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</thead>
<tbody>
<tr>
<td>UFF</td>
<td>GC3</td>
<td>3.3</td>
<td>0.078</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Urinary nitrogen</td>
<td>0.05</td>
<td>0.006</td>
<td>0.36</td>
</tr>
<tr>
<td>UFF + UFE</td>
<td>GC3</td>
<td>15.5</td>
<td>0.001</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Urinary nitrogen</td>
<td>0.11</td>
<td>0.003</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Plasma leptin</td>
<td>−7.3</td>
<td>0.001</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>5α-THF/THF</td>
<td>−35.7</td>
<td>0.057</td>
<td>0.09</td>
</tr>
</tbody>
</table>

a Stepwise multiple regression results (models a priori adjusted for GC3).

b Total $R^2$ for significant predictors ($P < 0.05$) without 5α-reductase index (5α-THF/THF).
TgAb (<60 kU/L), but recovery test results were abnormal (58%, range 80%–120%). The patient underwent modified right-neck dissection, which provided histological confirmation of PTC metastasis in 4 of 34 dissected lymph nodes. The Tg and TgAb measurement and recovery tests were performed on a fully-automated Immulite 2000 (DPC) system. In this case, because of the mismatch between serum and FNAB-Tg concentrations and the low Tg recovery, we further searched for interference on the serum Tg assay. First, we retested Tg, Tg-recovery, FNAB-Tg, and TgAb using sensitive immunoassays (Tg-plus and Dyno-test Tg, BRAHMS). BRAHMS assay results indicated that Tg was pathologically increased and Tg-recovery was within the reference interval (Table 1). On the basis of these results we hypothesized that antibodies other than TgAb were causing interference. We then measured serum Tg on the Immulite platform after treating serum samples in a heterophilic blocking tube (Scantibodies Laboratory). After incubation in a heterophilic blocking tube, a Tg increase to 18.2 μg/L was found, confirming interference by HAb leading to a false-negative result.

TgAb interference is a major pitfall, leading to a falsely low or negative result in approximately 20% of DTC patients sera at the time of cancer diagnosis in the US. The frequency is lower in Europe and declines after thyroidectomy. Use of the TgAb immunometric assay is strongly recommended to screen for interferences in patients with DTC, but the usefulness of the recovery-test is under debate. However, because no consistent correlation pattern has been demonstrated between different Tg and TgAb immunoassays, undetectable TgAb in apparently TgAb-negative sera should be regarded with caution (2). In these cases a recovery test should be considered, especially if Tg testing did not fit the clinical picture (3). In our patient, the low recovery signaled interference on the Tg assay, with increased TgAb undetected by 2 different immunoassays. Generally the HAb binds to both the capture and detection antibody, simulating the presence of analyte in its absence and resulting in a false-positive result or a falsely increased measurement if the analyte is present. Preisnser et al. (2) evaluated 1106 serum Tg samples and detected HAb interferences in approximately 3% of the specimens tested, without falsely low or negative results; however, samples with Tg concentrations <1 μg/L were excluded from the study. In some cases, HAb binds only to the capture (or detection) antibody, leading to falsely low or negative analyte measurement results (4). As shown in the present case (for the 1st time, to our knowledge) HAb may also interfere with testing by decreasing the measured Tg, leading to false-negative results. The interfering antibody cannot be differentiated with the blocking tube, but animal immunoglobulins added to serum should be useful. Interestingly, HAb did not significantly interfere with Tg measurement in FNAB washing fluid. Similarly, the Tg-FNAB did not appear to be substantially affected by TgAb, probably because of the very high Tg concentrations in the needle-washing fluids (5).

In conclusion, the Tg assay is a cornerstone in DTC follow-up and management. Tg measurement is significantly limited in TgAb-positive patients, however, and HAb may increase or, as shown here, even decrease the measured Tg. Ultrasound examination is of pivotal importance in the management of DTC neck recurrences, and in this case was supported by FNAB, allowing a definitive diagnosis. Thus effective management of DTC will continue to depend on multidisciplinary collaboration, especially for high-risk or relapsing patients.

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Table 1. Tg, TgAb, and Tg-recovery evaluation by two different immunometric methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Tg</th>
<th>TgAb</th>
<th>Tg-recovery</th>
<th>Tg-FNAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immulite</td>
<td>&lt;0.9</td>
<td>&lt;35</td>
<td>58%</td>
<td>950.70</td>
</tr>
<tr>
<td>BRAHMS</td>
<td>12.4</td>
<td>&lt;60</td>
<td>98%</td>
<td>&gt;250</td>
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</table>
Laboratory Medicine: Value for Patients Is the Goal

To the Editor:

Bossuyt et al. (1) make interesting points regarding the major transformations in laboratory medicine in recent decades. They also underline the new competencies required by laboratory professionals to address current challenges, including the need to give “comprehensive consultative support” to clinicians. I would like to emphasize that the evolution of clinical laboratories into knowledge services is the key not only for their survival but, even more relevant, for improving patient safety.

A body of evidence has been collected in the last few years to demonstrate that a large percentage of laboratory errors occurs in the pre- and postanalytical steps (2), in particular at the beginning (pre-analytical phase) and at the end (post-analytical phase) of the total testing process. These errors are especially related to requests for an inappropriate test and errors in patient and sample identification, data interpretation, and actions taken on behalf of the patients. In particular, studies during the past 30 years have documented that clinicians ignore or overlook 25%–60% of abnormal routine and STAT tests (2); a more recent study demonstrated that a much smaller but still high percentage (3.5%) of abnormal results are not documented in the patient medical report (3).

Clinical laboratories should, therefore, assume some responsibility for the whole cycle of testing, including appropriateness of test request and interpretation, so that the reported data may result in effective patient management and, ultimately, satisfactory clinical outcomes (4). This responsibility, however, should be achieved only through a close liaison with and the involvement of clinicians and other healthcare professionals in the quality loop. Commoditization, outsourcing, and the establishment of megalaboratories that simply spew out analytical results undermine the effective governance of the total testing process, and thus increase the number and risk of errors in laboratory medicine.

The report by Bossuyt et al. (1) is also welcome because it may prevent the eventual rapid spreading of a new condition, the “Alamo syndrome”. Under the increasing economic pressure affecting the delivery of laboratory services, some professionals seem to seek refuge in a closed environment (i.e., consolidated structures and megalaboratories), focusing on the mere reduction of the cost per test and on other efficiency indicators within the laboratory walls. Yet, as we all know, no one defending the Alamo survived the siege. The survival of clinical laboratories should be achieved by opening effective channels in the clinical context in which tests are required and used; laboratory services should be planned, commissioned, and delivered within a network, as part of an integrated healthcare system, and in relation to their impact on the patient’s “journey”. A focus on these processes should lead to the revision of and improvement in the timeliness, efficiency, and effectiveness of delivering laboratory information. This process must occur in the wider context of integrated clinical pathways for meeting real clinical needs, including more multidisciplinary efforts, identifying opportunities for cooperative approaches for delivering clinical information (e.g., integrating information from laboratory and imaging techniques), and streamlining training and education toward these aims.

Four main principles should therefore underpin changes in the delivery of laboratory services: (a) the goal must be value for patients and for public health; (b) laboratory services must be organized around medical conditions and care cycles; (c) clinical and economic outcomes must be measured; and (d) competition between different laboratories must hinge on the best possible quality and value in patient care, not solely upon cost per test result.

Finally, some comments should be made regarding the statement “services offered by clinical laboratories are more and more perceived as homogeneous, because many tests are performed on automated instruments using commercially available reagents” (1). As a matter of fact, current evidence shows that analytical quality is still a major issue, and data from internal quality control and from external quality assessment programs do not consistently demonstrate that the results from clinical laboratories meet evidence-based quality specifications (2). Therefore, technology is a fundamental, but not a unique feature in assuring analytical quality. Laboratorians should make more efforts to implement and communicate to clinicians the value of quality specifications, their relationships with clinical needs, and their relevance for appropriate interpretation and use of laboratory results (5).

The pathway for the future is to stimulate competition based on value and to provide knowledge services with analytical excellence as their mandatory starting point. If this mission is achieved, the interpretation and utilization of laboratory information will be more effective, and the Alamo syndrome will be banished.

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References

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Nanotechnology and Immunoassay

To the Editor:
The roots of nanotechnology can be traced back to colloid science in the mid-19th century. Francesco Selmi, often credited with the first true research in the field, studied the behavior of various metallic and acidic emulsions in the 1840s (1). Shortly thereafter, Michael Faraday and Thomas Graham, two scientific pioneers known primarily for their work with electricity and diffusion of gases, respectively, contributed further to the analysis of nanoparticulate suspensions, i.e., colloids. Faraday’s studies in electrochemistry naturally led him to the first experiments with colloidal gold (2), and Graham coined the term “colloid” after noting that certain substances showed slow diffusion rates through porous membranes (3).

Early applications of nanostructures in analysis included the use of colloidal gold and silver (i.e., nanosized gold and silver particles) as part of histologic stains (4). Since then, the scope of analytical applications of nanotechnology has increased considerably (5). One branch of analysis in which nanotechnology has had a significant impact is immunoassays.

A broad range of nanostructures (e.g., spheres and tubes) are being used to push the detection limits and throughput of immunoassay technology. Metallic and semiconductor nanoparticles, for example, are gaining popularity as highly sensitive labels (e.g., in fluorescent detection and Raman tagging), and magnetic nanoparticles are being used as solid supports for immunoassays. In addition, the fabrication of nanobarcodes structures has recently opened the door to the development of high-throughput multiplexed assays. Finally, the optimized design of self-assembled monolayer and nanoimprinted substrate surfaces has yielded precisely organized surface-immobilized antibodies, further improving the performance of immunoassays.

We have compiled a database of recent references that encompasses the body of research that lies at the intersection of nanotechnology and immunoassays, covering the period from 2005 to mid-2007. This database is intended to provide a comprehensive view of nanotechnology’s expanding role in immunologic techniques as well as spark a more focused effort in this area of study.


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References

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Use of a Bone Marrow Transplantation Model System to Demonstrate the Hematopoietic Origin of Plasma S100B mRNA

To the Editor:
The analysis of circulating RNA in plasma opens up new possibilities for the noninvasive monitoring of a variety of physiological and pathological conditions (1). In this study, we demonstrate the usefulness of a bone marrow transplantation (BMT) model system for ascertaining the tissue origin of plasma RNA species. These data were generated as part of a project to develop plasma nucleic acid markers for brain injury. Because brain injury, such as stroke, involves cell death and disruption of the blood-brain barrier, we hypothe-
A heterozygous C/T shift to homozygous T/T shift was observed in cases 9 and 15. A homozygous T/T to C/C shift was observed in cases 2, 3, and 11. A homozygous T/T to homogygous C/C shift was observed in cases 12, 14, and 16.

Table 1. S100B genotypes of BMT recipients and donors.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>DNA specimen from recipient’s pre-BMT PBMCs</th>
<th>RNA specimen from recipient’s post-BMT plasma</th>
<th>Both DNA and RNA specimens from recipient’s post-BMT buffy coat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CT</td>
<td>CT</td>
<td>CT</td>
</tr>
<tr>
<td>2*</td>
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</table>

* Cases were considered informative because they showed distinct S100B genotypes between the donor and recipient before BMT. Post-BMT shifting of recipient’s plasma S100B genotype was observed in all of these cases: A heterozygous C/T to homozygous C/C shift was observed in cases 2, 3, and 11. A heterozygous C/T to homozygous T/T shift was observed in cases 9 and 15. A homozygous T/T to heterozygous C/T shift was observed in cases 12, 14, and 16.

Numerous reports indicate that S100 calcium binding protein B (S100B) is released after brain injury and its concentrations in serum are significantly correlated with injury severity and outcome (2). To investigate whether S100B mRNA might be detectable in the plasma and might serve as a marker for brain injury, we used a real-time reverse transcriptase PCR assay to measure plasma S100B mRNA concentrations in 67 stroke patients and 16 healthy age- and sex-matched individuals. Among the stroke patients, stroke was diagnosed as ischemic in 48 patients and hemorrhagic in 6 patients, and 13 patients had no acute changes on computed tomography or magnetic resonance neuroimaging. Median patient scores for the National Institutes of Health Stroke Scale (NIHSS) and Glasgow Coma Scale (GCS) were 5 (range 0–34) and 15 (range 4–15), respectively. The median time from symptom onset to blood sampling was 11 h (range 1–23.8 h). Eight stroke patients died during hospitalization or within 30 days after discharge. Plasma S100B mRNA concentrations, however, showed no statistically significant differences in stroke patients compared with controls (P = 0.38, Mann–Whitney test), and plasma S100B mRNA concentrations in stroke patients showed no statistically significant correlation with NIHSS or GCS scores. These unexpected results prompted us to investigate whether the detected plasma S100B mRNA was indeed derived from the brain. Previous data from a BMT model have demonstrated that hematopoietic cells are important contributors of plasma DNA. It is possible that hematopoietic cells might also contribute to the detected plasma S100B mRNA through illegitimate transcription (3).

We thus proceeded to use the BMT model to test whether the hematopoietic system might indeed contribute significantly to plasma S100B mRNA. Genotypes of S100B mRNA molecules in plasma of recipients after BMT were compared with those before BMT as well as with the donor genotypes. Contribution of plasma S100B mRNA by blood cells could be inferred if the transcripts in plasma of recipients bore the donor genotypes among the informative donor-recipient pairs, in which the donor and recipient had different S100B genotypes before BMT.

The local clinical research ethics committee approved the study. Twenty-three myeloablative BMT patients who attended the Department of Paediatrics of the Prince of Wales Hospital, Hong Kong, were recruited, and informed consent was obtained from either the patients or responsible guardians. Archived pre-BMT peripheral blood mononuclear cells (PBMCs) of the recipients were retrieved for DNA extraction. A post-BMT blood sample (5 mL EDTA) was withdrawn from each patient and then centrifuged at 100 g for 10 min at 4 °C. Plasma and buffy coat samples were then collected separately. Plasma samples were filtered with a 5-μm filter to remove any residual blood cells. Buffy coat samples were centrifuged at 230 g for 5 min at 4 °C, and any residual plasma was removed.

The methods of nucleic acid extraction and single nucleotide polymorphism (SNP) analysis of RNA molecules, i.e., RNA-SNP, were as previously described (4, 5). In brief, the analysis was based on the detection by mass spectrometry of an SNP within an RNA transcript after a primer extension reaction. The extension products for each allele demonstrated distinct masses that could
be resolved by MALDI-TOF mass spectrometry.

A highly polymorphic C/T SNP, rs9722 (minor allele frequency 0.43), located within the coding region of S100B was selected from the Sequenom RealSNP Assay Database. Post-BMT plasma S100B genotypes of recipients were determined from the RNA specimens that were extracted from the recipients’ post-BMT plasma samples. Pre-BMT S100B genotypes of the recipients were determined from the DNA specimens that were extracted from the archived pre-BMT PBMC samples of the recipients. The S100B genotypes of the bone marrow donors were determined from both DNA and RNA specimens that were extracted from the post-BMT buffy coat samples of the corresponding recipients.

As shown in Table 1, 8 of 23 donor-recipient pairs were informative. The S100B mRNA genotypes in the plasma of these recipients were altered after BMT and became identical to those of the corresponding donors.

Our study has clearly demonstrated that after BMT the S100B mRNA in recipient plasma switched to the donor’s genotype. Thus, plasma S100B was predominantly of hematopoietic origin and could not be used as a brain-specific plasma marker. Hence, caution is required in the interpretation of the presumed tissue origin of plasma RNA markers. The BMT model described here can provide a valuable confirmation step for showing or excluding the hematopoietic contribution of a plasma RNA marker.

Grant/funding support: This work was supported by funding from the Li Ka Shing Foundation and the Chinese University of Hong Kong research direct grant account 4450148. Y.M.D.L. is supported by the Chair Professorship Scheme of the Li Ka Shing Foundation. Financial disclosures: T.H.R., N.Y.L.L., R.W.K.C., and Y.M.D.L. have filed patent applications on aspects of plasma nucleic acid analysis.

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