found in benign breast diseases *(25)*. In addition, the presence of PSA (in particular in the uncomplexed form) in NAF is in accordance with the high concentration of enzymes, proteases, and some biochemical analytes that may be prone to metabolic alterations in ductal epithelial cells of women with breast diseases *(11)*. In another study we described at the electron microscopic level the epithelial cells present in NAFs, giving evidence for highly metabolizing cells (Malatesta et al., submitted for publication). Accordingly, the intense anti-PSA labeling on ribosomes, sites of protein synthesis, strongly suggests that NAF cells are able to synthesize this protease; moreover, the presence of some labeling along the cell surface could indicate a secretory activity. The small vacuoles containing PSA could represent storage/complexation sites of this protease, although we cannot exclude that they can be removed by exocytosis. Therefore, breast epithelial cells present in NAFs could be a candidate source of PSA found in nipple aspirate extracellular fluid; similarly, in type I breast gross cysts, the apocrine cells occurring in the cystic fluid are responsible for PSA production and secretion *(24)*.

The production and secretion of several proteases (including PSA) and biologically active compounds by female breast apocrine cells in association with a lack of physiologic control of the secretion/reabsorption mechanism *(11)* and/or with prolonged exposure to several biologically active compounds through the autocrine/paracrine mechanism *(13)* could, with age, make the highly metabolizing apocrine cells prone to premalignant transformation *(26)*. However, the clinical significance of the PSA molecular forms and of its production/secretion by breast epithelial cells with respect to breast cancer risk should be evaluated cautiously *(17, 18)*.

This work was supported in part by a grant from the Assessorato alla Sanità, Regione Marche, Italy.

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Comparison of Immunoreactivity of Five Human Cardiac Troponin I Assays toward Free and Complexed Forms of the Antigen: Implications for Assay Discordance, *Pradip Datta,[1*] Kimberley Foster,[1] and Amitava Dasgupta[2]* ([1] Bayer Diagnostics, 333 Conney St., E. Walpole, MA 02032; [2] University of Texas-Houston, Houston, TX 77030; * author for correspondence: fax 508-660-4591, e-mail pradip.datta@chirondiag.com)

Troponin, consisting of three components, troponin C (TnC), troponin I (TnI), and troponin T (TnT), is a major component of the structural proteins involved in striated and cardiac muscle contraction *(1, 2)*. TnI and TnC bind tightly to each other in the presence of Ca^{2+} with an association constant, K_a, of $\sim 10^8$-10^9 L/mol *(1–3)*. TnT binds to both TnC and TnI, although less weakly than the binding between TnC and TnI. The cardiac isoforms of TnI (cTnI) and TnT are structurally different from the corresponding skeletal isoforms, and therefore they have recently established themselves as biochemical markers of myocardial damage *(4–7)*.

The currently available cTnI assays produce differing

results *(8–10)*. One important reason is that the assays may differ in their responses to the various isoforms of cTnI present in circulation or in biochemical preparations. In addition to "free" cTnI (I) and its binary and ternary complexes with TnC and TnT (IC or ICT) *(8, 11, 12)*, cTnI may exist in phosphorylated *(13)*, oxidized *(14)*, and proteolytically degraded *(8, 11, 12)* forms. The latter modified forms of cTnI also may exist as binary or ternary complexes. All of these forms may have different recognition patterns in different immunoassays. The predominant form of cTnI in acute myocardial infarction (AMI) patients is the binary complex IC *(8, 11)*. Samples treated with a Ca^{2+} chelator such as EDTA would contain mostly free cTnI because chelation of Ca^{2+} disrupts IC and ICT complexes.

We explored the possibility that the sera from patients with different pathological conditions may contain different isoform distributions of cTnI, thus generating discordant results among assays that recognize the isoforms differently. Here we report the immunoreactivity of five commonly used commercial cTnI immunoassays (Bayer ACS:180®, Dade Stratus®, Beckman Access®, Behring OPUS®, and Abbott AxSYM®) toward the two major forms of the analyte: free cTnI and its binary complex, IC.

Free cTnI (I) and its binary complex (IC), both isolated from human heart, were obtained from Scripps Laboratories. Their concentrations, determined by protein assay (Bio-Rad), were provided by the vendor and then converted to molar cTnI concentrations, using molecular weights of 21 000 and 35 000 for I and IC, respectively. Normal human serum, obtained from Scantibodies, tested negative for cTnI in all five cTnI assays.

Buffered stock solutions of both I and IC, as obtained from the vendor, and the serum were mixed to produce 0, 5, 10, and 20 μg/L solutions of cTnI, which were analyzed by five cTnI immunoassays: ACS:180, Stratus, AxSYM, OPUS, and ACCESS. Manufacturer-suggested assay protocols and platforms were used for all assays. All five assays use a "sandwich" method, where the analyte is sandwiched between capture and label antibodies, and thus the generated signal is directly proportional to the concentration of TnI in the sample. Details of the assays are in listed in Table 1.

When the results of the methods listed in Table 1 (y) were compared with the Stratus (x), the linear correlation slopes were 0.14 for ACCESS, 1.13 for ACS:180, 1.43 for OPUS, and 3.42–5.14 for AxSYM. We calculated the OPUS (y)/Stratus (x) slope as the ratio of slopes for (ACS:180/Stratus) and (ACS:180/OPUS). The two different slopes of AxSYM (y) vs Stratus (x) were obtained from different studies: 3.42 from the package insert of the AxSYM kit, and 5.14, from our own studies *(15)*. The AMI cutoff values varied in slightly different order: ACCESS (0.15 μg/L) < ACS:180 ~ Stratus (1.5 μg/L) < AxSYM ~ OPUS (2.0 μg/L). The ACCESS and AxSYM assays differed by a factor of as much as 36-fold (5.14/0.14). The other three assays agreed more closely (± 25%). Various survey data for cTnI also indicate similar differences among the assay results *(16)*. Many of the difference are probably contributed by standardization. As noted by other researchers *(8–10)*, such interassay differences underscore the urgent need for universal standardization for this important analyte.

Fig. 1 presents the comparison of immunoreactivity of the five assays to IC (Fig. 1A) and I (Fig. 1B). The assays have different orders of immunoreactivity toward free cTnI and IC complex: ACCESS < Stratus < ACS:180 < OPUS < AxSYM for IC; and Stratus < ACCESS < OPUS < ACS:180 < AxSYM for I. The order of immunoreactivity for IC was similar to the order of their responses in the method comparison study (Table 1). However, whereas the ACS:180, ACCESS, and AxSYM cTnI assay

Table 1. Comparison of the five cTnI immunoassays used in this study.

	ACS:180	Stratus	AxSYM	OPUS	ACCESS
Format/label type	Sandwich/ chemiluminescent	Sandwich/ fluorescent	Sandwich/microparticle enzyme-fluorescent	Sandwich/ fluorescent	Sandwich/ chemiluminescent
Manufacturer	Bayer Corp. Diagnostics Division Walpole, MA	Dade Behring Miami, FL	Abbott Diagnostics Abbott Park, IL	Dade Behring Westwood, MA	Beckman Chaska, MN
Platform run on:	ACS:180 analyzer	Stratus II analyzer	AxSYM analyzer	OPUS Magnum analyzer	ACCESS analyzer
Capture antibody	Two different mouse monoclonal antibodies	Mouse monoclonal antibody[a]	Mouse monoclonal antibody	Goat polyclonal antibody[a]	Mouse monoclonal antibody[a]
Label antibody	Goat polyclonal antibody	Mouse monoclonal antibody	Goat polyclonal antibody	Goat polyclonal antibody	Mouse monoclonal antibody
Sample size, μL	100	200	220	250	50
Range, μg/L	0.15–50	0.3–50	0.3–50	0.5–150	0.03–50
Cutoff for AMI, μg/L	1.5	1.5	2.0	2.0	0.15
Method comparison slope (y/x)	1.13[b]	3.42[c]	0.22[e]	0.79[e]	0.14[d]
	(ACS:180/Stratus)	(AxSYM/Stratus)	(ACS:180/AxSYM)	(ACS:180/OPUS)	(ACCESS/Stratus)

[a] The capture antibody is different from the label antibody, recognizing different sites on the cTnI molecule.
[b–d] From the package inserts for the kits; [b] ACS:180; [c] AxSYM; and [d] ACCESS.
[e] Dasgupta et al. *(15)*.

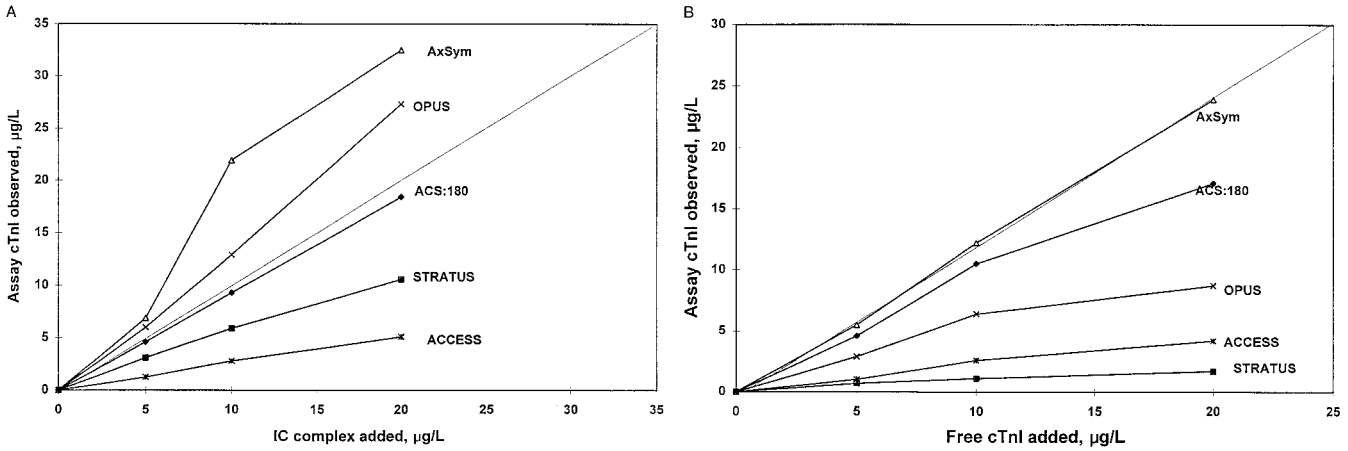

Fig. 1. Results of five different cTnI assays compared for serum samples supplemented with binary IC complex (*A*) or free cTnI (*B*).

In *A* and *B*, the *solid line* is the line of identity. (*A*), the ACS:180 assay is closest to the line of identity, AxSYM and OPUS are *above* the line of identity, and ACCESS and Stratus are *below* the line of identity. The linear correlation slopes of the five assay results with respect to the added cTnI concentrations are: ACS:180, 0.92 (r = 0.999); Stratus, 0.53 (r = 0.998); AxSYM, 1.68 (r = 0.978); OPUS, 1.37 (r = 0.999); and ACCESS, 0.26 (r = 0.999). (*B*), the ACS:180 assay is closest to the line of identity, AxSYM is *above* the line, and the other assays are *below* the line of identity. The linear correlation slopes of the five assay results with respect to the added cTnI concentrations are: ACS:180, 0.86 (r = 0.992); Stratus, 0.08 (r = 0.978); AxSYM, 1.20 (r = 0.999); OPUS, 0.43 (r = 0.968); and ACCESS, 0.21 (r = 0.990).

responses to I remained similar to their responses to IC, the Stratus and OPUS assays behaved quite differently to I than to IC, producing responses to I that were 6.41 and 3.19 times less than IC (as determined the ratio of slopes, IC/I; Fig. 1). The ACS:180 cTnI assay showed the best "equimolarity" (i.e., the ability to recognize I and IC equally well), with a IC/I slope ratio of 1.07. The slope ratios for the ACCESS and AxSYM assays were 1.23 and 1.4, respectively.

Our observations agree well with those of Wu et al. *(8)* who compared nine different immunoassays for cTnI toward synthetically prepared oxidized or reduced forms of I or IC. When they compared the more common oxidized forms of IC and I, six assays in their study were approximately equimolar, two others responded to IC better than to I by ~1.5-fold, and one assay responded to IC ~3.2-fold more than to I.

Because the binary IC complexed form of cTnI is believed to account for ≥90% of cTnI in the serum of most AMI patients *(8, 11, 12)*, the ACS:180 and Stratus assays show close agreement among most samples (Table 1). However, if the complexed form of cTnI is released into the circulation only after extensive damage to myocytesand the subsequent necrosis of cardiac muscle, the appearance of IC or ICT complexes in the circulation could take several hours to days, thus delaying the confirmation of AMI diagnosis. On the other hand, 5–6% of the total cardiac cTnI exists as a free form in the cytoplasm *(17)*. If the free cTnI is released faster than IC complex from damaged myocytes, initial cTnI concentrations may contain a higher percentage of free cTnI. These samples may show a higher frequency of discordance between the equimolar and nonequimolar cTnI assays.

We think that the differential immunoreactivity of cTnI assays to its isoforms may explain some cases of assay discordance. A possible example can be found in a method comparison study with samples from many categories of cardiac patients *(18)*. When the Stratus, OPUS, and ACCESS assay results for 138 such samples were compared, a poorer linear regression coefficient (r = 0.774) was found between Stratus and ACCESS than between Stratus and OPUS (r = 0.92) or OPUS and ACCESS (r = 0.90) *(18)*. Our finding that the order of equimolarity between these three assays is ACCESS (most equimolar) > OPUS > Stratus (least equimolar) may explain those data. An assay that detects both forms of cTnI equally well could be an advantage over a method that detects only one form of cTnI because the presence of any form of cTnI in serum is indicative of cardiac damage.

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Glutamic Acid Decarboxylase Antibodies in Screening for Autoimmune Diabetes: Influence of Comorbidity, Age, and Sex on Specificity and Threshold Values, *Manou R. Batstra,[1*] Arianne van Driel,[1] Jacob S. Petersen,[2] Cees A. van Donselaar,[3,7] Maarten J. van Tol,[4] G. Jan Bruining,[1] Diederick E. Grobbee,[5] Thomas Dyrberg,[6] and Henk-Jan Aanstoot[1,8]* (Departments of [1] Pediatrics, [3] Neurology, and [5] Epidemiology and Biostatistics, Erasmus University, 3015 GE Rotterdam, The Netherlands; [2] The Hagedorn Research Institute, Gentofte DK2820, Denmark; [4] Department of Pediatrics, Leiden University Medical Center, Leiden 2333 ZA, The Netherlands; [6] Diabetes Immunology, Novo Nordisk A/S, Bagsværd DK2880, Denmark; [7] Hospital St. Clara, Department of Pediatrics, Rotterdam 3078 HT, The Netherlands; [8] IJsselland Hospital, Department of Pediatrics, Capelle a.d. IJssel 2906 ZC, The Netherlands; * address correspondence to this author at: Department of Immunology, Ee 893, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands; fax 31-10-4087038, e-mail Batstra@immu.fgg.eur.nl.)

Antibodies against the 65-kDa isoform of glutamic acid decarboxylase (GAD_{65}) can be applied as a predictive tool for childhood type-1 diabetes *(1–6)* and to facilitate the differential diagnosis of diabetes in adults *(7–9)*. However, the sensitivity and specificity of GAD antibody screening have not been fully characterized, and the positive predictive value of screening varies from 20% to 70%, depending on the strategy applied and the population studied *(2, 3, 5–7, 9–12)*. The current study aims to identify factors that may lead to false-positive results in GAD antibody screening.

Previously, it has been demonstrated that the GAD antibody frequency in mixed connective tissue disease and stiff-man syndrome is increased, although not all patients who suffer from these diseases and are positive for GAD antibodies develop type-1 diabetes *(13, 14)*. GAD is expressed in the islets of Langerhans, neuronal tissue, the ovaries, and the testes *(15, 16)*. Similar to the above-mentioned examples, comorbidity involving these tissues may lead to GAD antibody formation but not to diabetes. Therefore, we compared the prevalence of GAD antibodies in patients with cystic fibrosis, epilepsy, Guillain-Barré syndrome, and premature ovarian failure to the prevalence in an unselected population of 1403 schoolchildren.

In addition, thresholds for positivity for GAD antibodies have generally been defined in children. These thresholds might not be applicable when testing for type-1 diabetes in adults. Therefore, we studied whether GAD antibody concentrations are correlated to age and sex, and whether adjustment of assay thresholds to include these variables may improve screening specificity.

The frequencies of positive results for GAD antibodies and the concentrations of GAD antibodies were established in a population of 1403 schoolchildren, ages 10–12 years, without chronic diseases *(17)*. During a 10-year follow-up, two of these children developed type-1 diabetes [ascertainment >96% *(18)*].

The influence of comorbidity on GAD antibody concentrations and the frequencies of positive results were studied in four patient populations. The subjects included 394 patients who participated in the Dutch study of epilepsy in childhood *(19, 20)*. These patients were eligible for the current study if the diagnosis was confirmed on the basis of electroencephalograms or therapy and sufficient serum for antibody analysis was available. Sera collected within 2 months after the presenting seizure (mean duration, 0.7 months; n = 228) and at the longest disease duration available of each patient (mean duration, 12.2 months; range, 2–50 months; n = 294) were analyzed separately. The diagnosis and development of diabetes during follow-up (5 years) were recorded from the medical records. Forty-three serum samples from 38 cystic fibrosis patients (collected in 1990–1992) were analyzed for GAD antibodies. In addition, we studied 30 patients with premature ovarian failure and 28 patients with Guillain-Barré syndrome (14 males; age range, 19–64 years). All patient sera were stored at −80 °C.

The influence of age and sex on GAD antibody concentrations and the frequencies of positive results were studied in 1287 individuals from the city of Zoetermeer, who participated in a study of cardiovascular risk factors. Sera were collected in 1976 and stored at −20 °C until testing. The population is described in detail elsewhere *(21)*. The demographic data of the populations are shown in Table 1.

The study protocols were approved by the appropriate medical ethics committees according to the Helsinki Declaration. Informed consent was obtained from all participants or their parents.

Sera were tested for GAD antibodies by radiobinding assay (RBA) *(13)* or immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Triton X-100 fraction of [^{35}S]methionine-labeled fetal rat