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One of the authors of the article cited above responds:

To the Editor:

There is a clear need for more accurate and efficient markers for the assessment of glomerular filtration rate (GFR). Because the introduction of various new therapies for treating cancer requires reliable assessment of a patient's renal function, the application of new markers in clinical practice is particularly important in oncology. Cystatin C has been proposed as a promising marker of renal functional impairment, having significantly better characteristics than creatinine, a marker commonly used for clinical determination of GFR *(1)*. However, the increasing evidence that overexpression of cysteine proteinases plays an active role in tumor progression *(2)* raises the possibility that their inhibitors, including cystatin C, may also be up-regulated in cancer patients. Indeed, in melanoma and colorectal cancer patients, we noticed significantly higher serum cystatin C than in controls *(3)*. Because serum creatinine, a rough indicator of GFR, was not changed, we raised the possibility of nonrenal effects on the serum concentration of cystatin C in patients with malignant disease. Of course, for definitive evidence, a reference GFR procedure needs to be used, but for the large number of patients and controls included in our studies (>600) this would have been impracticable.

The aim of our Letter to the Editor in *Clinical Chemistry* was to highlight the increased cystatin C in serum of patients with two kinds of malignancy, a result that is relevant not only to cancer researchers, but also to clinical chemists. We hope that our work will stimulate further studies to establish definitively the behavior of cystatin C in patients with malignant disease and the potential of cystatin C for assessing GFR.

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Digibind and Free Digoxin

To the Editor:

In their article investigating digoxin measurements in the presence of Digibind®, Ocal and Green *(1)* conclude that "useful free digoxin concentrations may be obtained for Digibind-treated patients using either the AxSYM or Stratus immunoassays. . . ". Yet they cite several references that report that digoxin concentrations measured with the Stratus® instrument were significantly higher than were free digoxin concentrations measured after ultrafiltration *(2–5)*. The AxSYM® assay has also been shown to yield higher results than ultrafiltration *(6)*. These studies contradict the assertion that the AxSYM and Stratus assays are measuring free digoxin concentrations. Nonetheless, one can agree that results from the Stratus II and AxSYM may be suitable for monitoring the course of Digibind treatment. To use these results most effectively, one must understand their limitations and the probable sources of the discrepancy with ultrafiltration results.

The results of previous investigations, as well as binding kinetics theory, suggest that these immunoassays will overestimate the free digoxin concentration. The extent of overestimation will vary with the extent of digoxin binding to albumin, with the assay measurement strategy, and with the absolute and relative amounts of digoxin and Digibind in the specimen. In most instances, the extent of the overestimation will be small enough that the result can be quite informative. In particular, these results set an upper limit on the concentration of pharmacologically active digoxin and can be used to rule out recurrent toxic concentrations.

One reason that the Stratus and AxSYM assay results exceed free digoxin concentration is that they are designed to measure total digoxin in routine specimens, including digoxin bound to serum proteins, principally albumin. Immunoassay antibodies have digoxin binding affinities significantly greater than that of albumin. Assay incubation times are sufficient for albumin-bound digoxin to dissociate fully, to bind to the assay antibodies, and to be measured. However, albumin-bound digoxin is not free digoxin and will not be measured after ultrafiltration. (It should be noted that any digoxin that is not bound to Digibind will exhibit the usual distribution between free and albumin-bound fractions.) If albumin-bound digoxin were the only source of discrepancy between the two types of measurement, it could be handled simply by using a reference interval for total digoxin [e.g., 1.0–2.6 nmol/L (0.8–2.0 ng/mL)] for the Stratus and AxSYM results and a reference interval for free digoxin [e.g., 0.8–2.0 nmol/L (0.6–1.6 ng/mL)] for ultrafiltration assays. However, the differences that have been observed are >25% and cannot be explained solely by albumin binding [normal range, 20% ± 5% *(7)*].

Another probable contributor to the observed differences is measurement by the immunoassays of some

of the digoxin that is bound to Digibind. There are theoretical reasons to expect that this will occur. According to the manufacturer, Digibind is a polyclonal mixture of antibody Fab fragments having a range of affinities, with dissociation constants (K_d) ranging from 10^{-9} mol/L to 10^{-10} mol/L (1–0.1 nmol/L) *(8)*. Immunoassay antibodies may be expected to have comparable or higher affinities because it would be difficult to accurately measure digoxin concentrations in the therapeutic range (1–2.6 nmol/L) if the affinity was lower. When a specimen containing digoxin-in-Digibind complexes is assayed, some of the bound digoxin will dissociate and be captured by the assay antibodies. The extent of transfer will be limited by the incubation time and the dissociation rates for the complexes. Studies of the antibody binding kinetics of small antigens indicate typical association rates of 10^7 to 10^8 L/mol-s *(9)*. This implies a dissociation rate of 1–10% per second for digoxin bound to Digibind fragments with a K_d of 10^{-9} mol/L, and 0.1–1% per second from fragments with a K_d of 10^{-10} mol/L. Because bound digoxin concentrations are often in the range of 50–100 nmol/L (40–80 ng/mL) after Digibind treatment *(5, 10, 11)*, only a small percentage needs be transferred to the assay antibodies to increase the apparent unbound digoxin concentration by a significant amount.

A third possible source of overestimation is the presence of empty Digibind fragments. Unless these are removed by pretreatment or an early wash step, they can be expected to bind some of the labeled digoxin tracer in the assay, thereby reducing the amount of tracer available to compete with unbound digoxin for assay antibody. If these Digibind-tracer complexes are subsequently removed, as occurs in the Stratus assay, this will yield less bound tracer and an increase in apparent digoxin. This effect would not be expected in the AxSYM assay because Digibind is removed by a wash step before tracer addition.

Other possible components of the discrepancy between immunoassay and ultrafiltration values could be underestimation of free digoxin by ultrafiltration methods because of differential ultrafiltration rates for digoxin and water molecules *(10, 12)*, nonspecific binding of digoxin to the ultrafiltration device *(13)*, or matrix effects from calibrators not prepared in serum ultrafiltrate *(11, 14)*. As Ocal and Green *(1)* note, the measurement of free digoxin by ultrafiltration has not been well-validated. Thus, it is not currently possible to estimate to what extent, if any, measurements after ultrafiltration underestimate actual free digoxin concentrations.

Currently, patients treated with Digibind can be monitored reasonably, using either the Stratus or AxSYM immunoassays or an ultrafiltration method free of matrix effects. It must be remembered that the immunoassays will overestimate free digoxin concentrations, whereas ultrafiltration may underestimate it. Occasionally, it might be appropriate to use both approaches to set upper and lower limits on the actual concentration.

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To the Editor:

Although we agree in general terms with the premise put forth by Dr. Rainey, we believe it is important to not lose sight of the fact that the various reasons proposed to account for the biases between the AxSYM and Stratus direct immunoassays and ultrafiltration, although plausible, are hypotheses, not proven observations. Further experimental work should be done to determine more definitively the root causes of these biases. As stated in our report *(1)*, and reiterated by Dr. Rainey in his letter, some of the biases can be accounted for in terms of albumin-bound digoxin (retained by ultrafiltration but measured by the direct method). This, without doubt, accounts for a significant portion of the bias.

On a more conceptual point, under physiological conditions, receptors to which digoxin binds have access to both free and albumin-bound digoxin because albumin-bound digoxin must,