Quantifying the Effects of Renal Impairment on Plasma Concentrations of the Neuroendocrine Neoplasia Biomarkers Chromogranin A, Chromogranin B, and Cocaine- and Amphetamine-Regulated Transcript

To the Editor:

Neuroendocrine neoplasia (NEN)\(^1\) accounts for 2% of all malignancies (1). Patients with NEN often present with nonspecific symptoms and thus represent a major diagnostic challenge. There are several circulating NEN biomarkers, and chromogranin A (CgA) is regarded as the gold standard (2). Chromogranin B (CgB) has been found to be a useful diagnostic addition to CgA measurements (3). The peptide product of cocaine- and amphetamine-regulated transcript (CART) is also increased in patients with NENs, particularly in those with pancreatic NENs (4).

Renal impairment or failure can increase circulating concentrations of CgA (2) and CART (4); however, CgB may be unaffected by mild renal impairment and is increased only in severe renal failure (3). The population most likely to be affected by NEN is also susceptible to renal impairment: Chronic kidney disease (CKD) occurs in approximately 10% of the population between 50 and 60 years of age, and the mean age of patients with a NEN diagnosis is 61 years (1, 5). We therefore examined the effect of varying degrees of renal impairment/failure on plasma concentrations of CgA, CgB, and CART in patients without NEN.

Ethics approval for this study was obtained from the Hammersmith and Queen Charlotte’s and Chelsea Hospitals Research Ethics Committee (04/Q0406/80). After informed written consent was obtained, 5 mL of blood was collected from 40 healthy volunteers. In addition, samples from 107 patients with different stages of renal impairment were obtained and irreversibly anonymized as per Royal College of Pathology, UK, guidelines (D035, September 2007). All blood samples were collected into EDTA-containing tubes and centrifuged at 10,000g for 10 min within 15 min of venipuncture. Plasma samples were then stored at −20 °C until analysis. CgA, CgB, and CART concentrations were measured by immunoreactivity (IR) (CgA-IR, CgB-IR, and CART-IR) with an in-house RIA (4) at the National Gut Hormone Specialist Assay and Advisory Laboratory, Imperial College Healthcare NHS Trust, UK.

To test for significant differences between healthy volunteers and individuals at different CKD stages, we analyzed the data by Kruskal–Wallis one-way ANOVA and adjusted for multiple comparisons with the Dunn test (Fig. 1).

Fig. 1. Plasma CgA concentrations as measured by IR (CgA-IR), CgB-IR concentrations, and CART-IR concentrations increase with decreasing eGFR.

One-way ANOVA with Dunn a posteriori test compares groups of patients in different CKD stages against healthy volunteers for each biomarker. NS, not statistically significant (\(P > 0.05\)); *\(P = 0.036\); **\(P = 0.026\); ***\(P < 0.001\).

Medians and interquartile ranges (error bars) are shown. Dashed line indicates upper reference limit for each biomarker.

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1 Nonstandard abbreviations: NEN, neuroendocrine neoplasia; CgA, chromogranin A; CgB, chromogranin B; CART, cocaine- and amphetamine-regulated transcript; CKD, chronic kidney disease; IR, immunoreactivity; CgA-IR, CgA as measured by IR; eGFR, estimated glomerular filtration rate.
tions were normal in patients with mild renal impairment (CKD stages I and II). Although there were no significant differences with respect to CgB-IR (P = 0.070) and CART-IR (P = 0.0858), CgA-IR was increased significantly (P = 0.036) in patients with eGFR values ≥60 mL·min⁻¹·(1.73 m²)⁻¹, compared with the healthy volunteers. These results are interesting because they suggest that although all 3 peptides are affected to varying degrees by renal failure, CgA-IR may increase even with mild renal impairment [eGFR ≥60 mL·min⁻¹·(1.73 m²)⁻¹]. Thus, CgB-IR may be a more reliable marker than CgA-IR in patients with mild renal impairment [eGFR >45 mL·min⁻¹·(1.73 m²)⁻¹]. CgB is larger than CgA and CART and therefore may not be as dependent on glomerular filtration, which may explain the fewer cases of increased concentrations of CgB-IR among patients with renal disease.

Interestingly, no patients with renal failure had CgA-IR or CART-IR concentrations >500 pmol/L or CgB-IR concentrations >250 pmol/L. Therefore, although the diagnostic sensitivity of these NEN markers is low at these higher cutoffs (CgA-IR, 20% at 500 pmol/L; CART-IR, 17% at 500 pmol/L; CgB-IR, 12% at 250 pmol/L), the diagnostic specificity for NEN diagnosis is very high (100% for all 3 biomarkers). Patients with concentrations above these cutoffs must be investigated for NENs, even in the presence of renal failure. Our results indicate the need for additional studies to determine the diagnostic cutoffs with optimal sensitivity and specificity for the neuroendocrine biomarkers to enable NEN diagnosis in renally impaired patients.

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References

Letters to the Editor:

Methotrexate is a potentially toxic folic acid antagonist that is widely used as an immunosuppressant and chemotherapeutic agent. After high doses (0.035–12 g/m²) are administered, methotrexate concentrations in the plasma or serum are carefully monitored so that the patient can be rescued, if necessary, with the proper dose of leucovorin, a folic acid analog that bypasses the important enzymes inhibited by methotrexate (1).

Recently, the manufacturer of the fluorescence polarization immunoassay we use for monitoring (TDx platform; Abbott Laboratories) announced its intention to move the assay to a different proprietary platform. In addition, the immunoassay is known to be nonspecific. For example, the assay strongly cross-reacts with diamino-5-methylpteroyl acid, a minor (<5%) endogenous methotrexate metabolite and the major (>98%) product of pharmacologic inactivation of methotrexate with carbapenemase G. As an alternative to reagent-dependent proprietary methods, a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method, with an improved specificity, can be used to measure methotrexate (2, 3).

Many laboratories, including our own, have replaced immunoassays for other immunosuppressants, which can also suffer from interferences, with laboratory-developed tests that use LC-MS/MS (4). In contrast to methotrexate, the number of samples processed for other immunosuppressants with the immunosuppressant assay is high enough to support the LC-MS/MS infrastructure in many laboratories. To avoid adding staff and instrumentation for a new methotrexate assay, we developed a method to quantify methotrexate and other immunosuppressants in the same assay.

Our current immunosuppressant work flow involves precipitating protein from whole blood with acetonitrile containing 100 mmol/L ZnSO₄ (from Thermo Fisher and Sigma-Aldrich, respectively) and LC-MS/MS analysis with a C18 column and a Quattro Micro mass spectrometer (Waters). Quantification is performed with cyclosporin D as an internal standard for cyclosporin A (Eton Biosciences) and ascomycin (Calbiochem) as an internal standard for sirolimus and tacrolimus (Eton Biosciences) (4). As a first step to determine whether we could simply add methotrexate, we identified the optimal multiple reaction monitoring transitions (455 → 308 and 458 → 311, [M+H]⁺ molecular ions) for methotrexate (MP Biochemicals) and its deuterated internal standard, methotrexate-d₃ (Toronto Research Chemicals), respectively. Unfortunately, methotrexate was not retained by the C18 solid phase. We tested other chemistries and determined that a Supelco C8 column (5 μm, 2 cm × 2.1 mm; Sigma-Aldrich) could resolve methotrexate and the other immunosuppressants and that one chromatographic method worked for all of the analytes of interest [0–0.4 min: 50% to 100% methanol in 2 mmol/L ammonium acetate, 1 mL/L formic acid, and water (J.T. Baker); 0.4–1.0 min: 100% to 50% methanol; and 1.0–3.5 min: equilibration and needle wash]. Unfortunately, the ZnSO₄ in the precipitation reagent, which is important for low precision and low limits of detection in the immunosuppressant assay, greatly increased the limit of detection for methotrexate. This effect may be due to inadequate chelation of the Zn²⁺ cation, which is likely accomplished by heme in whole-blood lysates (5). After further experimentation, we determined that we could use our current precipitating reagent for the immunosuppressants and a 50–50 mixture (by volume) of methanol (Fisher Scientific) and acetonitrile as the precipitating reagent for methotrexate. With 2 independent sample-preparation reagents, we used a separate 6-point calibration curve for each sample type.

In the final assay for methotrexate, serum (100 μL) was added to 400 μL precipitation reagent. After vortex-mixing for 5 min and centrifugation for 10 min at 13 000g, we injected 20 μL of the supernatant into the LC-MS/MS system. For methotrexate, the assay was linear from 0.01 μmol/L to 10 μmol/L. The intraassay CV (n = 20) was 1.6% and 3.5% at 0.77 μmol/L and 0.08 μmol/L metho-