Glomerular Filtration Rate Is a Confounder for the Measurement of Soluble Mesothelin in Serum

To the Editor:

The mesothelin gene encodes a 71-kDa precursor protein that is subsequently cleaved into a soluble megakaryocyte potentiating factor and a membrane-bound part, mesothelin. Mesothelin can be shed as “soluble mesothelin” or “soluble mesothelin-related protein” (SMRP), which is an approximately 40-kDa biomarker of malignant mesothelioma, an asbestos-related cancer (1). Mesothelioma typically occurs in middle-aged men, and the renal function or the glomerular filtration rate (GFR) can occasionally decrease in these patients and in individuals at risk of developing mesothelioma. Renal impairment leads to the accumulation of low molecular weight proteins in the blood [e.g., cystatin C (13 kDa) and β-trace protein (BTP) (23–29 kDa)], and these proteins have been used as markers of the GFR. Such renal impairment can also cause the accumulation of soluble mesothelin, as has previously been reported for a limited number of cases (2). Our aim, therefore, was to thoroughly investigate the impact of renal function on serum SMRP concentrations.

We enrolled 66 individuals (49 men and 17 women; median age, 58 years; range, 24–80 years), who were referred for measurement of 51Cr-EDTA clearance to estimate the GFR. We excluded patients with asbestos-related diseases/malignancies, patients with endometrial, ovarian, cervix, lung, breast, pancreatic, or gastrointestinal cancer, and patients with leukemia, because these conditions can overexpress mesothelin (3, 4). The study was approved by the ethics committee of both participating hospitals, and participants gave written informed consent. In addition, we analyzed GFR data from 51 chemotherapy-naive mesothelioma patients (47 men and 4 women; median age, 63 years; range, 45–79 years) who had undergone treatment at our institution between 2003 and 2008.

We used cystatin C, BTP, and creatinine as serum markers to assess the GFR. The plasma clearance of 51Cr-EDTA was used as a reference standard for the GFR and was measured as previously described (5) with adjustment to a standard body surface area of 1.73 m². Creatinine was measured by a rate-blanked compensated Jaffe method on a Modular P analyzer (Roche Diagnostics). BTP and cystatin C were measured by latex-enhanced nephelometry on a Behring BN II analyzer (Siemens Diagnostics). Serum SMRP was measured with MESOMARK™ ELISA kits (Cisbio/Fujirebio Diagnostics) (4). The GFR of mesothelioma patients was measured with the isotope-dilution mass spectrometry–traceable Modification of Diet in Renal Disease (MDRD) equation (6).

A Spearman rank analysis showed a significant correlation (P < 0.001) between the GFR and the reciprocal of the concentrations of cystatin C (r = 0.785), BTP (r = 0.770), and creatinine (r = 0.818). The reciprocal of the SMRP concentration was significantly correlated with the GFR (r = 0.494; P < 0.001) and the reciprocals of the concentrations of cystatin C (r = 0.370; P = 0.002), BTP (r = 0.402; P = 0.001), and creatinine (r = 0.462; P < 0.001). The median SMRP concentration was 1.25 nmol/L (range, 0.30–5.28 nmol/L), and the SMRP concentration in 24 of the 66 patients was >1.5 nmol/L, the manufacturer’s cutoff for differentiating healthy controls from mesothelioma patients (4)—a false-positive rate of 36% (Fig. 1).

We then categorized the GFRs of the 66 patients referred for 51Cr-EDTA clearance testing into 5 stages: stage 1, normal [≥90 mL·min⁻¹·(1.73 m²)⁻¹]; stage 2, mildly decreased [60–89 mL·min⁻¹·(1.73 m²)⁻¹]; stage 3, moderately decreased [30–59 mL·min⁻¹·(1.73 m²)⁻¹]; stage 4, severely decreased [15–30 mL·min⁻¹·(1.73 m²)⁻¹]; and 5, renal failure [<15 mL·min⁻¹·(1.73 m²)⁻¹]. Falsely increased SMRP concentrations were observed in none of the 11 stage 1 patients, but false positives were observed in 7 (32%) of 22 stage 2 patients, in 10 (40%) of 25 stage 3 patients, in 6 (86%) of 7 stage 4 patients, and in the single stage 5 patient. The mean SMRP concentration of the patients with a nonpathologic GFR was significantly different from that of the patients with a decreased GFR (P < 0.001, ANOVA).

Of the 51 mesothelioma patients, 22 (43%) had a typical renal function, 24 (47%) showed a mildly decreased GFR, and 5 (10%) had a moderately decreased GFR. The majority of the mesothelioma patients thus had a decreased GFR and therefore were susceptible to an accumulation of SMRP in the serum.

Theoretically, the corrected SMRP concentration (SMRPcorr) (in nanomoles per liter) based on a patient’s renal impairment can be derived from the equation for the linear regression of the reciprocal of the SMRP concentration on the GFR for the 66 patients: 1/SMRP = 0.268 L/nmol + 0.01 · GFR (r² = 0.255; P < 0.001; Fig. 1). Therefore, the measured SMRP (SMRPmeas) can

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1 Nonstandard abbreviations: SMRP, soluble mesothelin-related protein; GFR, glomerular filtration rate; BTP, β-trace protein; MDRD, Modification of Diet in Renal Disease.
be corrected for the difference in the patient’s GFR from a reference GFR \([120 \text{ mL} \cdot \text{min}^{-1} \cdot (1.73 \text{ m}^2)^{-1}]\):

\[
\text{SMRP}_{\text{corr}} = \left( \frac{1}{\text{SMRP}_{\text{meas}}} + \frac{120 - \text{GFR}}{100} \right)^{-1}
\]

In conclusion, the serum SMRP concentration is significantly correlated with the GFR, which makes the GFR a confounder in the application of the SMRP ELISA kit. Furthermore, the majority of the investigated mesothelioma patients had a decreased GFR. Therefore, caution is advised when interpreting test results in individuals with an impaired renal function, e.g., in screening high-risk populations, because reductions in the GFR can produce falsely increased SMRP concentrations.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors’ Disclosures of Potential Conflicts of Interest:** Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

**Employment or Leadership:** None declared.

**Consultant or Advisory Role:** None declared.

**Stock Ownership:** None declared.

**Honoraria:** None declared.


**Expert Testimony:** None declared.

**Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Assay Imprecision and 99th-Percentile Reference Value of a High-Sensitivity Cardiac Troponin I Assay

To the Editor:

We undertook a performance evaluation and determination of the 99th-percentile reference value for the Siemens cTnI-Ultra with a fully characterized population that had undergone noninvasive cardiac imaging. Permission for the study was obtained from the local research ethics committee. Participants >45 years of age were randomly selected from the practice lists of 7 representative local community practices; 1392 individuals from the general population were invited. Demographic data were collected from the participants by questionnaire. Heart rate and blood pressure measurements (mean of 2 readings) and spirometry, electrocardiography, and echocardiography evaluations were performed. For measurements of fasting serum glucose, creatinine, and cardiac troponin, venous blood was collected into Becton Dickinson serum separator tubes and centrifuged. The serum was removed and stored at -70 °C. Frozen samples were thawed to room temperature, mixed, and centrifuged before analysis. The left ventricular ejection fraction was calculated quantitatively with Simpson’s apical biplane method (1). The left ventricular mass was calculated with the Devereux-modified American Society of Echocardiography equation (2). Valvular regurgitation and stenosis were assessed qualitatively on a 5-point scale. Diastolic heart failure was defined according to the European Study Group on Diastolic Heart Failure guidelines. Healthy individuals were defined as persons from the general population with the following: no history of vascular disease, diabetes mellitus, hypertension, or heavy alcohol intake; not receiving cardiac medication; blood pressure <160/90 mmHg (mean of 2 readings); fasting blood glucose <6 mmol/L; estimated creatinine clearance [calculated by the modification by diet of renal disease equation corrected to a reference creatinine method (3)] >60 mL·min⁻¹·(1.73 m²)⁻¹; and a nonpathologic echocardiogram [defined as follows: no significant valvular heart disease (grade 1–2), a left ventricular ejection fraction >50%, a left ventricular mass index <134 g/m² for men and <110 g/m² for women, no diastolic heart failure or regional wall-motion abnormalities, an isovolume relaxation period of <90 ms, an E/A ratio >1.0 at <50 years of age or >0.5 at ≥50 years, and an E-wave deceleration time of <220 ms at <50 years of age or <280 ms at ≥50 years].

All analyses were performed on the ADVIA Centaur (Siemens Healthcare Diagnostics) with the manufacturer’s recommended protocols. The stated detection limit is 0.006 μg/L, and upper limit is 50 μg/L. The claimed 10% CV is 0.03 μg/L, with a 99th percentile value of 0.04 μg/L. Total imprecision was assessed by following CLSI protocol EP15-A with serum pools prepared from sera of known high cardiac troponin concentrations that were adjusted by dilution with serum considered to be troponin free according to the cTnI-Ultra assay. The pools were then stored frozen at -20 °C until use. Seven concentrations were measured 4 times daily for 5 days. The samples were stored at 4 °C between runs.

![Fig. 1. Mean CVs and 95% CIs for cardiac troponin I (cTnI) concentrations.](image-url)
Statistical analyses were performed with the Analyse-it add-in for Microsoft Excel (version 2.12; Analyse-it, www.analyse-it.com).

Fig. 1 summarizes assay imprecision. By interpolation, we estimated that the 10% CV occurred at 0.045 µg/L. Of the 1392 individuals invited to participate, 699 (50.2%) reported for screening; 336 were male. The median age was 58.0 years (interquartile range, 53–71 years). After the exclusion of ineligible individuals, we collected samples from the reference population of 309 individuals (127 men, 182 women). The median age was 53 years (range, 45–80 years; interquartile range, 49–62 years). The age distributions of the male and female participants were not statistically different for either the screening set or the reference set. There was no association between cardiac troponin I concentration and blood pressure. The troponin values of the male and female participants were not significantly different, and there was no correlation between troponin concentration and age for either the overall population or the reference set. Troponin was undetectable (no signal detectable above background) in 25 individuals and had a calculated value between 0 µg/L and 0.006 µg/L in 144 individuals; hence, 165 (53.4%) of the 309 individuals could be considered to have no measurable troponin. The 99th percentile was 0.039 µg/L.

This study used a randomly selected population of ostensibly healthy individuals, which was then screened to exclude any possibility of active cardiovascular disease. The one previous evaluation of the Siemens Ultra method showed both an age and sex dependence of the troponin values (4). That study did not use imaging and obtained a higher value for the 99th percentile. A comparable study that also measured cardiac troponin I by a high-sensitivity method demonstrated similar findings (5). In contrast, however, our study with a highly selected subset of individuals defined by nonpathologic results in cardiac imaging analyses found no effect of sex on the 99th percentile and obtained a lower value for the 99th percentile.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Cross-Reactivity of Naloxone with Oxycodone Immunoassays: Implications for Individuals Taking Suboxone®

To the Editor:

In 2002, the US Food and Drug Administration approved the use of the sublingual tablet, Suboxone® (Reckitt Benckiser Pharmaceuticals), to treat opioid dependence. The formulation is available at 2 doses, 2 or 8 mg (free base) buprenorphine hydrochloride and 0.5 or 2 mg (free base) naloxone hydrochloride dihydrate (1). The combination product is designed to decrease the potential for intravenous abuse, since parenteral administration may precipitate opioid withdrawal (2).

To monitor compliance, physicians request urine testing of buprenorphine and often additional testing to ensure no illicit drug use. A client recently requested multi-

References

analyte testing of patients taking Suboxone. Initial testing suggested a response with the oxycodone assay. We hypothesized this response was due to the presence of naloxone. To test this hypothesis, we (a) assayed a 40-μg/L buprenorphine calibrator with the oxycodone assay and (b) assayed drug-free urine samples that had been supplemented with increasing concentrations (100–10,000 μg/L) of naloxone (Cerilliant Corporation) and naloxone glucuronide (Sigma–Aldrich).

We evaluated 2 enzyme immunoassays: the homogeneous enzyme immunoassay (HEIA) oxycodone (Immunalysis Corporation) and the DRI® oxycodone assay (Microgenics/Thermo Fisher Scientific). On the Hitachi 717 analyzer, the HEIA demonstrated 100% cross-reactivity with oxymorphone at 100 μg/L (3), and the DRI assay demonstrated 103% cross-reactivity with oxymorphone at 300 μg/L and <0.1% cross-reactivity with noroxycodone and noroxymorphone at 50,000 μg/L and 500,000 μg/L, respectively. The Microgenics package insert indicated that 200 mg/L naloxone would test negative with a 100-μg/L cutoff for the DRI assay (4). No information was provided in the Immunalysis package insert regarding cross-reactivity with naloxone. Each kit contained oxycodone calibrators at 0, 100, 300, 500, and 1000 μg/L. A cutoff of 100 μg/L was used. Reagents were tested in the semiquantitative mode on a Dade Behring/Siemens Viva-E instrument. The instrument was set up according to the program notes provided by the manufacturers.

Buprenorphine at 40 μg/L did not cross-react with either oxycodone assay. Fig. 1 illustrates the responses obtained by both assays with increasing concentrations of naloxone and naloxone glucuronide. The highest response was obtained with the HEIA for both analytes. The DRI response did not approach the cutoff, but the HEIA reached the cutoff at concentrations near 2000 μg/L for naloxone and 5600 μg/L for naloxone glucuronide. This response may be due to antibody specificity and/or to sampling parameters of the instrument. Urinary naloxone concentrations in patients taking Suboxone have not been widely reported. Hull et al. (5) suggested that typical total naloxone concentrations are <100 μg/L because of the low bioavailability of naloxone in this formulation. In 7 samples that the authors judged to be adulterated or substituted, the total naloxone concentration in urine ranged from <100 μg/L to 15,155 μg/L (5). The authors speculated that the high concentrations observed in 5 samples were due to attempts to dissolve the tablet directly in the urine sample.

In our study of 39 buprenorphine-positive urine samples, the oxycodone HEIA was positive in 27 cases (69%), and the DRI assay was positive in 2 cases. The 2 DRI cases (also positive by the HEIA) were assayed in house by solid-phase extraction and subsequent GC-MS analysis for oxycodone (limit of detection, 250 μg/L), and none was detected. Three cases (including the 2 tested in house) were sent to NMS Labs for testing. Total naloxone was quantified by liquid chromatography–tandem MS, and total oxycodone and total oxymorphine were quantified by GC-MS. Oxycodone and oxymorphine were not detected at a reporting limit of 100 μg/L. Each of the 3 urine samples was positive for total naloxone, however, with measured concentrations of 220, 820, and 580 μg/L. According to our study, these concentrations were not high enough alone to account for the positive findings. This finding suggests that another substance or substances, including unidentified metabolites, were the major contributors to the positive results. For example, naloxone metabolism also produces normaloxone (noroxymorphone) and naloxol.

It is important that users of this methodology be aware of the cross-reactivity of naloxone and naloxone glucuronide with the Immunalysis oxycodone HEIA and, to a lesser extent, with the
Microgenics DRI oxycodone assay to prevent false accusations against patients taking Suboxone. Urine from these patients may produce positive results in oxycodone assays at low cutoff concentrations because of the presence of a combination of naloxone and unidentified compounds. A noteworthy finding is that if the cutoff concentration of the oxycodone assay had been raised from 100 μg/L to 300 μg/L, only 2 patients (5%) tested with the HEIA and no patients tested with the DRI assay would have been reported as positive.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: No authors declared any potential conflicts of interest.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

A Simple Homemade Apparatus for Real-Time Visualization of Nucleic Acid Electrophoresis That Saves Time, Optimizes Separation, and Reduces Chemical Risk

To the Editor:

Agarose slab gel electrophoresis continues to provide a low-cost alternative and a valuable complement to more sophisticated techniques for estimating the size of DNA molecules after restriction enzyme digestion, for evaluating PCR products, and for separating restricted genomic DNA or RNA before Southern or northern blot analysis (1, 2).

With traditional apparatus, the electrophoresis run must be interrupted before the agarose gel can be moved onto a UV transilluminator (wavelength approximately 300–360 nm), which is connected to a conventional or digital camera and placed in a dark environment for best results. This procedure is repeated until a satisfactory separation is achieved.

We have devised a simple, easily reproducible device (Fig. 1) that contains the following main components: (a) an electrophoresis chamber; (b) 2 UV light–emitting lamps (302 nm), which are placed below the plastic plate holding the gel; and (c) a commercial Web camera connected to a personal computer. The device can be opened to pour or remove a gel and to fill the sample wells. It is configured for connection to an external power supply and is equipped with a safety switch that shuts off the power when the device is opened. The apparatus was built with commercially available materials and may be easily reproduced at a moderate cost. Technical drawings, a list of materials used, and relative costs are available free of charge from the corresponding author.

The assembly of these components into a single unit makes our device different from the traditional apparatus; however, its key feature is the part of the electrophoresis chamber holding the gel tray, which is made of UV-transparent Plexiglas (4 mm; Rohm and Haas). This simple modification, combined with the positioning of the UV lamps under the electrophoresis chamber and the availability of a digital camera inside the apparatus, makes it possible to visualize and record separations of nucleic acids without interrupting the run and having to move the gel from the chamber.

Our apparatus has 3 substantial advantages compared with traditional devices. First, the visualization and recording of results is shortened and simplified. Digitalizing of the electrophoretic separation is accomplished by simply turning on the video camera and capturing the image. This procedure takes only a few seconds. Second, the separation of DNA molecules is easily optimized. In fact, DNA can be visualized at any moment of the electrophoresis run without interrupting it, allowing
selection of the best electrophoretic separation among those recorded. Third, the chemical risks to the operators are reduced. By making unnecessary any movement of the gel in and out of the chamber, use of our apparatus minimizes laboratory contamination and operator contact with the dangerous chemical agents added to the gel (e.g., ethidium bromide), loaded with the samples under analysis (e.g., SYBR® Green I) to make DNA visible under a UV source, or used to denature RNA (formaldehyde). Furthermore, the described apparatus may provide a basis for further configurational developments. In this regard, the possible implementation of recently developed DNA-detection systems based on UV light– or ultrabright green light–emitting diodes could provide further advantages in terms of sensitivity, throughput, and resolving power (e.g., (3)).

A commercial alternative to our apparatus is the FlashGel® system from Lonza Rockland (www.lonza.com). Compared with the FlashGel system, our apparatus has some substantial advantages; in particular, it is possible to carry out prolonged runs for increased separation of DNA fragments through the use of larger gels [140 mm (length) × 150 mm (width) × 20 mm (height) vs 70 mm (length) × 84 mm (width) × 20 mm (height)] and allowing a free choice of agarose type and concentration for better adaptation of a given protocol to the range of molecular weights of the molecules to be separated; however, the electrophoresis time required for satisfactory separation with our device is at least 4-fold longer than for the

![Different views of the apparatus for nucleic acid electrophoresis.](img)

A list of materials used (with relative costs) and technical drawings are available free of charge from the corresponding author upon request. PC, personal computer.
commercial device. This disadvantage is partially offset by the fact that our gels can accommodate 80 samples per run (compared with 34 for the FlashGel device). Furthermore, our method is markedly less expensive than the FlashGel device with respect to both the apparatus (613 vs 991 euros) and the agarose gels (0.08 vs 0.37 euros; cost per sample for electrophoresis on a 22-g/L NuSieve® agarose gel from Lonza Rockland).

Our apparatus is currently being used for various applications, including checking the size and quality of DNAs extracted from biological materials (e.g., blood and saliva), PCR products of mitochondrial DNA before sequencing, and RFLP typing of polymorphisms within the IRS1 (insulin receptor substrate 1) and MCM6 (minichromosome maintenance complex component 6) genes (4, 5).

This newly designed apparatus can be easily reproduced and used in laboratories where analytical and preparative agarose slab gel electrophoresis of nucleic acids is carried out. It has substantial advantages in terms of saving time, optimizing separations, and reducing chemical risks to the operators.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.

Consultant or Advisory Role: None declared.

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: Ministero dell’Istruzione, dell’Università e della Ricerca, PRIN project protocol no. 2007TXX33X; Istituto Italiano di Antropologia; and University of Rome “La Sapienza.”

Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Previously published online at
DOI: 10.1373/clinchem.2009.125609

1 Nonstandard abbreviations: BNP, B-type natriuretic peptide; NT-proBNP, amino terminal fragment of the BNP prohormone.

Reference Values for Plasma B-Type Natriuretic Peptide in the First Days of Life

To the Editor:

The clinical relevance of B-type natriuretic peptide (BNP)1 and the amino terminal fragment of its prohormone (NT-proBNP) as biomarkers in pediatric heart disease have recently been shown (1, 2). BNP and NT-proBNP concentrations are dependent on age and sex, at least in adults (3). Because commercial methods are affected differently by the presence in plasma of several peptides derived from the degradation of intact prohormone and BNP (3), little if any agreement exists among reported reference intervals, especially those used for infants during the first days of extrauterine life (1).

Data are scarce regarding the reference values for BNP and NT-proBNP in infancy (1, 2, 4). Recently, Nir et al. (2) summarized NT-proBNP concentrations measured with an electrochemiluminescence immunoassay method in 690 healthy individuals (47% males) with ages ranging from birth to 18 years, including 127 newborns in the first week of life (43 in the first 2 days). The NT-proBNP concentrations were highest in the first days of life, and then showed a marked decline. Concentrations in males and females differed only for children age 10–14 years.

Large reference value studies for BNP for newborns and infants have not been reported. To assess the reference values for BNP in the first days of life, we measured the peptide concentrations in residual EDTA plasma from blood samples.
(0.2–0.4 mL) collected from a superficial venous vessel of the heel in 126 apparently healthy newborns (females 57, males 69) throughout the first 7 days of extrauterine life. We measured peptide concentrations by using a fully automated immunoassay platform (Triage BNP reagents, Access Immunoassay Systems, REF 98200, Beckman Coulter). Moreover, we obtained EDTA plasma samples from 33 healthy infants (females 15, males 18) with ages ranging from 1–6 months. Blood was collected from healthy newborns and infants who underwent routine screening for genetic disorders (for newborns) or an endocrine work-up (for infants). All newborns were delivered at term (between 35 and 42 weeks of pregnancy) and presented at birth with body weight ranging from 2.5–4.1 kg and with an Apgar score ≥8; 24.6% of deliveries were by cesarean section. In infants, clinical examination results excluded the presence of acute illness (i.e., infections or electrolyte imbalance), and laboratory test results were within the reference intervals. We obtained parental informed consent for all newborn and infant study participants.

To minimize degradation of plasma BNP (3), immediately after withdrawal of blood samples we put them into disposable polypropylene tubes containing EDTA (1 g/L of plasma). Plasma samples were rapidly separated by centrifugation (2400 g) for 15 min at 4 °C and then assayed as soon as possible (no more than 4 h after blood withdrawal). The analytical characteristics and performance of the Access immunoassay method used in this study for the measurement of BNP were previously evaluated in our laboratory (5).

Plasma BNP concentrations were highest in the first 2 days of life (1-way ANOVA after logarithmic transformation of original data, P < 0.0001), with a progressive decline in the next days and weeks (first 2 days: mean 282.4 ng/L, SD 169.3 ng/L, median 243.5 ng/L, 90th percentile 468.7 ng/L, n = 66; third and fourth day: mean 193.0 ng/L, SD 141.9 ng/L, median 169.0 ng/L, 90th percentile 348.0 ng/L, n = 40, P < 0.0230 by Scheffé test after 1-way ANOVA compared to the 2 first days; from the 5th to the 7th day: mean 102.6 ng/L, SD 168.2 ng/L, median 45.0 ng/L, 90th percentile 259.5 ng/L, n = 20, P < 0.0001 compared to the 2 first days; from 1–6 months: mean 21.5 ng/L, SD 10.0 ng/L, median 23.0 ng/L, 90th percentile 37.2 ng/L, n = 33, P < 0.0001 compared to the 2 first days) (Fig. 1). BNP values were not sex dependent (males: mean 179.7 ng/L, SD 168.0 ng/L, median 147.0 ng/L, n = 87; females: mean 187.3 ng/L, ng/L, SD 184.1 ng/L, median 155.5 ng/L, n = 72; P = 0.9909 by t-test after logarithmic transformation of original data). There was no difference in BNP values in infants who were born with vaginal vs cesarean deliveries.

Our data for BNP, the biologically active hormone, are in agreement with the results reported by Nir et al. (2) for NT-proBNP, which is an inactive peptide with a longer plasma half-life. Both BNP and NT-proBNP plasma concentrations are highest in the first 2 days of life, with a progressive decrease in the next days and weeks. Soldin et al. (4) reported obtaining higher values when they measured BNP by a point-of-care testing method in EDTA whole blood samples during the first month of life. It is well known that BNP and NT-proBNP values are strongly method dependent (3); consequently, clinicians should take into
account the method used for the measurement of BNP and NT-proBNP when comparing our values to those found in their own laboratory.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: No authors declared any potential conflicts of interest.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Previously published online at DOI: 10.1373/clinchem.2009.126847