ARTICLES

High-Throughput Urinary Neopterin-to-Creatinine Ratio Monitoring of Systemic Inflammation

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Background: Systemic inflammation is a marker of ill health and has prognostic implications in multiple health settings. Urinary neopterin is an excellent candidate as a nonspecific marker of systemic inflammation. Expression as urinary neopterin-to-creatinine ratio (UNCR) normalizes for urinary hydration status. Major attractions include (*a*) urine vs blood sampling, (*b*) integration of inflammation over a longer period compared with serum sampling, and (*c*) high stability of neopterin and creatinine.
Methods: A high-throughput ultraperformance LC-MS method was developed to measure neopterin and creatinine together from the same urine sample. The assay was applied in several clinical scenarios: healthy controls, symptomatic infections, and multiple sclerosis. Area under the curve was compared between weekly and monthly sampling scenarios. Analysis of a single pooled sample was compared with averaging results from analysis of individual samples.
Results: The assay has excellent intraassay and interassay precision, linearity of dilution, and spike and recovery. Higher UNCR was demonstrated in female vs male individuals, older age, inflammatory disease (multiple sclerosis), and symptomatic infections. In healthy controls, fluctuations in inflammatory state also occurred in the absence of symptomatic infection or other inflammatory triggers. Analysis of a single pooled sample, made up from weekly urine samples, integrates inflammatory activity over time.
Conclusions: UNCR is a useful biomarker of systemic inflammation. The method presented offers simplicity, speed, robustness, reproducibility, efficiency, and proven utility in clinical scenarios. UNCR fluctuations underline the importance of longitudinal monitoring, vs a single time point, to capture a more representative estimate of an individual's inflammatory state over time.

IMPACT STATEMENT

Longitudinal monitoring of systemic inflammatory status is important to follow activity of chronic inflammatory disease in an individual over time, or response to treatment. A high-throughput ultraperformance LC-MS method was developed to measure urinary neopterin-to-creatinine ratio (UNCR), with excellent intraassay and interassay precision, linearity of dilution, and spike and recovery. The assay was applied in several clinical scenarios, including healthy young and older individuals, symptomatic infections, and multiple sclerosis. We demonstrate fluctuations in UNCR that underline the importance of longitudinal monitoring, vs a single time point, to capture a more representative estimate of an individual's inflammatory state.

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The role of systemic inflammation in determining outcome in a variety of health settings is increasingly recognized. These range from frailty in normal community-dwelling adults (*1*) to common conditions such as hearing loss (*2*), cardiovascular disease (*3*), dementia (*4*, *5*), postoperative delirium (*6*), and cancer (*7*). Hence, there is a need for a practical and robust way to measure systemic inflammation serially and noninvasively.

The cytokine cascade is central to systemic inflammation, and a pivotal cytokine appears to be interferon-γ (IFN-γ)[3], which can cross-talk with other cytokines in a variety of ways (*8*) and act as a master checkpoint regulator for many cytokines (*9*). Although for these reasons IFN-γ is a very good candidate as a marker of systemic inflammation, it has several disadvantages from a technical point of view. It is rapidly degraded and requires blood sampling, and receptor binding makes its measurement in a soluble phase difficult to relate to its biological effect.

Specific aromatic pteridines called neopterins are produced by monocytes and macrophages upon stimulation with IFN-γ, with reasonable specificity for IFN-γ vs other cytokines (*10*). Myeloid cells can convert guanosine triphosphate to 7,8-dihydroneopterin triphosphate, but the conversion of the latter to tetrahydrobiopterin is inefficient because of a high ratio of guanosine triphosphate cyclohydrolase I to 6-pyruvol-tetrahydropterin synthetase, which occurs after IFN-γ stimulation (see Fig. 1 in the Data Supplement). This results in production of a high amount of 7,8-dihydroneopterin after IFN-γ stimulation, which is not seen in any other cell type examined, conferring cellular specificity (*11*). Most 7,8-dihydroneopterin is oxidized to D-erythro-neopterin [2-amino-4-hydroxy-6-(D-erythro-1',2',3'-trihydroxypropyl)-pteridine] (*12*); a variable small amount undergoes epimerization to L-threo-neopterin [L-threo-6-(l',2',3'-trihydroxypropyl)-pterin; see Fig. 1 in the online Data Supplement] (*13*). Both neopterin isomers (D-erythro-neopterin and L-threo-neopterin), collectively referred to as neopterin from here on, are excreted into urine. When L-threo-neopterin is detectable in urine, its ratio to D-erythro-neopterin is roughly 1:10, but this ratio varies (*14*). Being stereoisomers, D-erythro-neopterin and L-threo-neopterin peaks are difficult to completely resolve (*15*).

Neopterin presents several advantages over IFN-γ as an inflammatory biomarker because it is more chemically stable, does not bind to receptors, and undergoes rapid renal clearance, meaning urine concentrations closely reflect neopterin biosynthesis. Urinalysis is particularly attractive because it is noninvasive, integrates inflammatory activity over a longer period compared with a blood sample, and, as a less protein-rich solvent, is less likely to suffer from matrix effects. Neopterin is expressed as urinary neopterin-to-creatinine ratio (UNCR, measured in micromole/mole), to normalize for hydration status.

UNCR is measurable at a basal level and increases nonspecifically in response to a number of immunological stimuli, such as bacterial, viral, and parasitic infections (*16*), and cancer and autoimmune diseases (*17*). It is an excellent candidate for 2 applications: (*a*) to follow activity of an existing chronic inflammatory disease in an individual over time, or in response to treatment, and (*b*) an integrator of systemic inflammation over time.

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[3] **Nonstandard abbreviations:** IFN, interferon; UNCR, urinary neopterin to creatinine ratio; UPLC-MS, ultraperformance liquid chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; AUC, area under the curve.

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Techniques used for the analysis of pteridines include ELISA (*18*) and capillary electrophoresis–laser-induced fluorescence (*19*, *20*). It would be advantageous if creatinine and neopterin are measured in the same run on the same platform, rather than using different kits at different times, to minimize variability. Methods that overcome this include HPLC (*21*) and ion-pair HPLC (*22*) using fluorometric detection for neopterin and UV detection for creatinine, and HPLC-MS (*23*). Ultraperformance LC-MS (UPLC-MS) presents an appealing alternative to these other methods owing to its high throughput and sensitivity. All published mass spectrometry-based neopterin measurement methods use D-erythro-neopterin as standard, but none have clarified whether L-threo-neopterin is also measured along with D-erythro-neopterin. Hence, we set out to develop and validate a robust high-throughput assay using UPLC-MS/MS and apply it to several clinical settings as exemplars.

MATERIALS AND METHODS

UPLC-MS/MS optimization

An ACQUITY UPLC was interfaced with a Waters Xevo triple quadrupole mass spectrometer equipped with an electrospray ionization probe, column oven, and autosampler (Waters). Reagents were purchased as described in Methods in the online Data Supplement. During sample preparation and analysis, urine samples were kept in the dark at 5 °C. UPLC and MS/MS were optimized as described in Methods in the online Data Supplement.

In the final optimized protocol, separation was achieved using a UPLC penta-fluoro-phenyl (1.7 µm, 2.1 × 100 mm, Waters) column kept at 24 °C and fitted with its equivalent guard column (1.7 µm, 2.1 × 5 mm, VanGuard, Waters). The primary mobile phase (A) was 0.2% formic acid, and the cosolvent (B) consisted of 0.2% formic acid in acetonitrile. Total run time was 8 min and included a 2.5-min gradient from 99% (A) to 93.5% (A) followed by a 0.5-min gradient to 100% (B), which was kept for 2 min before a 0.5-min gradient back to starting conditions (99% A) for 2.5 min, as shown in Table 1 in the online Data Supplement. Injection volume was set at 10 µL for neopterin analysis and 5 µL for creatinine analysis. Using this method, creatinine showed low retention, eluting just after the solvent front at 0.9 min, and D-erythro-neopterin eluted at 1.8 min (Fig. 1A). Similar retention times were seen in urine (Fig. 1B).

After separation, the compounds were monitored in scheduled multiple-reaction monitoring mode with positive electrospray ionization. Injection parameters consisted of cone energy = 30 V and collision energy = 20 V. The multiple-reaction monitoring transitions were 114.1 > 44.1 *m/z* for creatinine, 117.1 > 47.1 *m/z* for creatinine-D_3, 254.1 > 206.1 *m/z* for D-erythro-neopterin and L-threo-neopterin, and 259.1 > 210.1 *m/z* for D-erythro-neopterin-$^{13}C_5$. Concentration of analyte was calculated by integrating the area under the peaks using MassLynx software (version 4.1, Waters).

Calibration curve preparation

Stock standard solutions of creatinine and creatinine-D_3 were made in milli-Q water. The D-erythro-neopterin and D-erythro-neopterin-$^{13}C_5$ solutions were made in 33 mmol/L ammonium hydroxide. Aliquots were stored at −80 °C.

For the calibration curve, creatinine (5 mg/dL) and D-erythro-neopterin (100 µg/L) stock solutions were serially diluted in starting conditions, i.e., 99% mobile phase (A) and 1% mobile phase (B). Solutions of creatinine-D_3 (2.5 µg) and D-erythro-neopterin-$^{13}C_5$ (50 ng) were added to make up the standard solutions to 1 mL. Standards were measured in duplicate at the beginning of each run and every subsequent 24 h. The calibration curves were constructed by plotting average ratio of the peak areas (creatinine/creatinine-D_3 or D-erythro-neopterin/D-erythro-neopterin-$^{13}C_5$) against concentration of analyte. A typical calibration curve

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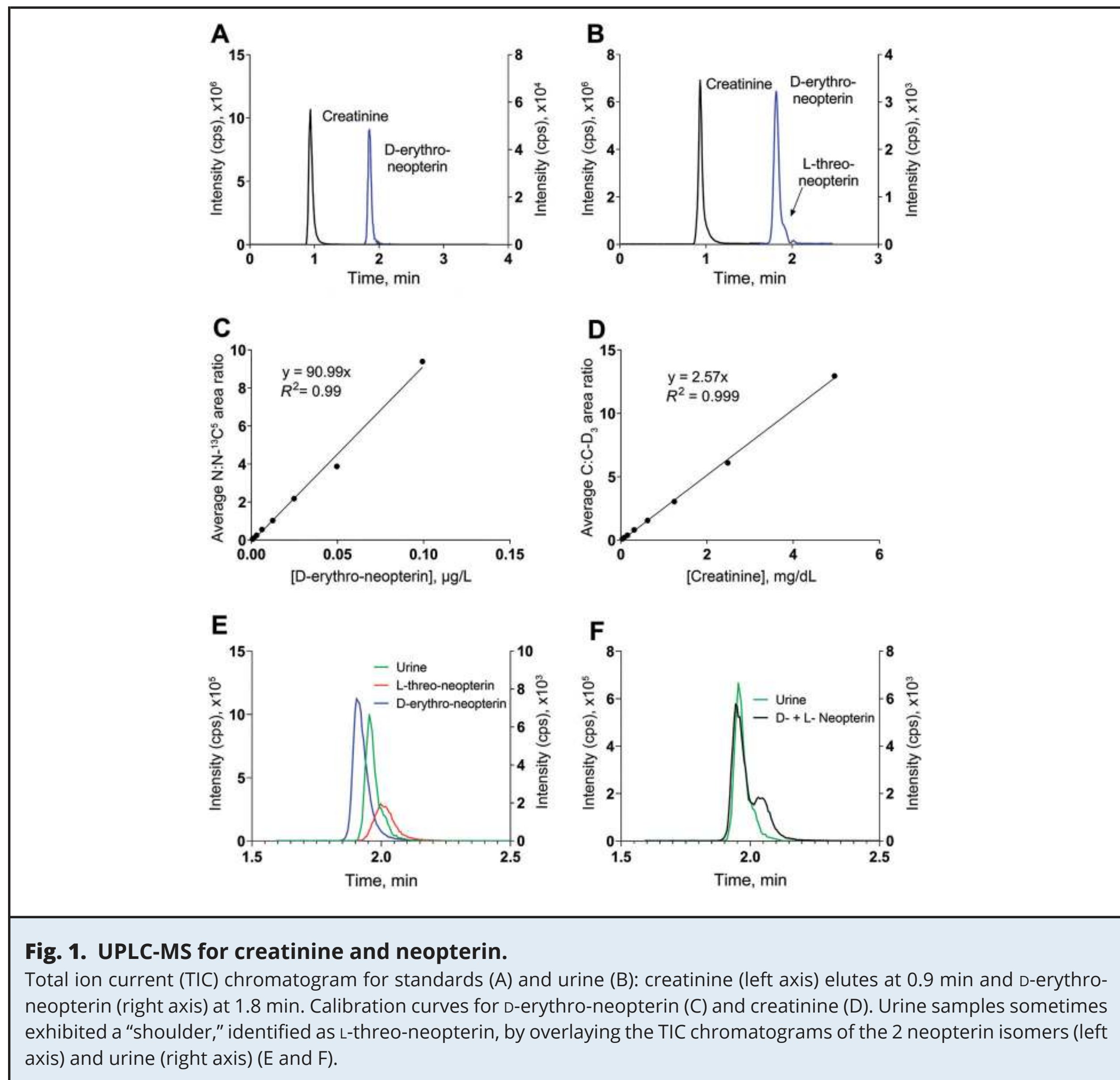

Fig. 1. UPLC-MS for creatinine and neopterin.
Total ion current (TIC) chromatogram for standards (A) and urine (B): creatinine (left axis) elutes at 0.9 min and D-erythro-neopterin (right axis) at 1.8 min. Calibration curves for D-erythro-neopterin (C) and creatinine (D). Urine samples sometimes exhibited a “shoulder,” identified as L-threo-neopterin, by overlaying the TIC chromatograms of the 2 neopterin isomers (left axis) and urine (right axis) (E and F).

for creatinine and D-erythro-neopterin is shown in Fig. 1, C and D.

Urine samples and analysis

Midstream urine was collected with informed consent from adults (National Research Ethics approvals 12/SC/0176 and 13/SC/0507, and institutional research ethics approvals ERGO 5562 and ERGO 7923). Participants were given kits and trained to shield urine from light. Urine was stored at −20 °C, then thawed and centrifuged at 10 °C and 2000*g* for 5 min. Aliquots were stored at −80 °C.

For analysis, individual urine aliquots were diluted 1:100 for neopterin and 1:500 for creatinine. Isotopically labeled internal standard was added to a total of 1 mL of solution. Samples were assayed in duplicate or triplicate. Urinary creatinine and neopterin concentrations were determined based on

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the calibration curve, expressed as microgram per liter and milligram per deciliter, respectively. UNCR was expressed in micromole per mole. A reference urine sample pooled from samples taken from 10 different individuals was used during each experiment to assess interassay precision.

Method validation

Primary validation was used because this is more robust than comparative validation. Validation included linearity, spike and recovery, precision, limit of detection (LOD), and limit of quantification (LOQ). Linearity was evaluated by plotting actual vs theoretical concentration of matrix-matched calibration points over a clinically relevant range (*23*).

Spike-and-recovery experiments were used to monitor matrix effects in healthy and diseased states (*24*). Urine samples from both healthy controls and progressive multiple sclerosis patients were spiked, in triplicate, with 3 concentrations of D-erythro-neopterin (100–5000 µg/L) or 3 concentrations of creatinine (5.0–300.0 mg/dL). Recovery was calculated using the following equation, using spike as denominator as per guidelines (*25*):

$$\%\ \text{Recovery} = \frac{[\text{measured concentration with spike}] - [\text{measured concentration without spike}]}{\text{spike concentration}} \times 100 \quad (1)$$

Method precision was assessed by interassay and intraassay CVs. Intraassay variation was established by replicate analyses (n = 10) of a pooled urine sample. Interassay variation was established by replicate analysis of the same pooled sample measured on separate days (n = 5).

Determination of LOD and LOQ is necessary only for analytes with concentrations close to zero (*26*); therefore, only the LOD and LOQ for neopterin were assessed, as creatinine is always present in urine at high concentrations. LOD and LOQ were calculated according to Eurachem guidelines (*25*) in which the SD of replicate measurements of samples with low levels of analyte was calculated and multiplied by 3 for LOD or 10 for LOQ (see Methods in the online Data Supplement for details).

Statistics

Analyses were performed in SPSS Statistics version 25 (IBM) or Prism version 7 (GraphPad Software). Descriptive statistics are shown as mean (SD). Nonparametric data were log-transformed. To compare groups in which age was a scalar covariate, analysis of covariance was used. Otherwise, 2-way ANOVA or Student *t*-tests were used. A *P* value of <0.05 was considered statistically significant.

RESULTS

Method validation

Matrix effects were tested in spike-and-recovery experiments. Ion suppression, often caused by matrix effects, can be a significant issue when using electrospray ionization (*27*). To assess a varied range of matrices, urine samples from both healthy controls and progressive multiple sclerosis patients were used. The influence of matrix effects is generally more pronounced at lower retention times because of the coelution of polar compounds commonly found in urine. Consequently, it was expected that creatinine, which elutes just after the solvent front, may show signs of ion suppression. Indeed, ion suppression accompanied by poor spike and recovery was seen in up to one-third of samples when using a 1:100 dilution. This phenomenon was abolished at a 1:500 dilution. As a result, for all further analysis, 2 sets of dilutions were used during the same run, namely, 1:100 for neopterin and 1:500 for creatinine. The average percentage recoveries for D-erythro-neopterin and creatinine in healthy control and progressive multiple sclerosis urine samples are shown in Table 1.

Both D-erythro-neopterin and creatinine exhibited excellent linearity ($R^2 > 0.99$) over a range of 0.5 to 140 µg/L for D-erythro-neopterin and 0.001 to 0.88 mg/dL for creatinine, corresponding to urinary

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Table 1. Average spike-and-recovery experiments for neopterin and creatinine in urine samples from healthy controls (HC) and progressive multiple sclerosis (pMS) patients.[a]

Sample type	Neopterin recovery, %					
	Low 100 μg/L		Medium 1000 μg/L		High 5000 μg/L	
	% Recovery	±SD	% Recovery	±SD	% Recovery	±SD
HC (n = 4)	102.4	5.5	104.7	2.8	101.4	2
pMS (n = 4)	99.2	11.5	101.9	2	100.9	0.5
All (n = 8)	100.8	9.2	103.3	2.8	101.1	1.5
Sample type	**Creatinine recovery, %**					
	Low 5 mg/dL		Medium 50 mg/dL		High 300 mg/dL	
	% Recovery	±SD	% Recovery	±SD	% Recovery	±SD
HC (n = 3)	97.3	4.3	96.2	1.5	109	4.6
pMS (n = 3)	99.7	12.2	103.1	3.6	101.7	5.5
All (n = 6)	98.5	9.2	99.7	4.4	105.4	6.3

[a] Results are shown for HC, pMS, and both groups combined.

concentrations of 50 to 14 000 μg/L and 0.5 to 450 mg/dL, respectively (see Fig. 2 in the online Data Supplement). These ranges were selected because they span the ranges encountered by us and others (*23*). Intraassay and interassay CVs (Table 2) indicate that the method is reliable and precise. The calculated values for the LOD and LOQ of D-erythro-neopterin were 0.3 μg/L and 1 μg/L, respectively.

L-threo-neopterin

The D-erythro-neopterin peak exhibited a small shoulder in some urine samples (Fig. 1B). We confirmed this as L-threo-neopterin (Fig. 1E and F). A spike-and-recovery experiment in urine was performed to show that total neopterin (D-erythro-neopterin + L-threo-neopterin) was reliably measured within the same peak, using the D-erythro-neopterin standard curve, in a more biologically relevant matrix. The spike (100 μg/L) was made up of 10% L-threo-neopterin and 90% D-erythro-neopterin so as to recapitulate a realistic ratio when L-threo-neopterin was present. Recovery was 106.48 ± 7.26 for healthy control urine samples (n = 4), 99.48 ± 13.64 for progressive multiple sclerosis urine samples (n = 4), and 102.98 ± 11.48 for all urine samples combined (n = 8).

Table 2. Measurement precision (percentage CV).

Compound	Precision	
	Interday %CV (n = 5)	Intraday %CV (n = 11)
Neopterin, μg/L	6.34	2.77
Creatinine, mg/dL	1.32	1.15
UNCR, μmol/mol	5.55	1.97

Clinical application

We assessed the use of UNCR in several clinical scenarios. Our aim was not to address the hypothesis that UNCR increases with inflammation because this has been tested before, but rather to demonstrate the usability of our assay. Two different sampling techniques were tested:

1. Single time-point urine samples in which UNCR was measured once (spot UNCR).
2. Urine samples taken serially over an extended period to determine UNCR area under the curve (AUC).

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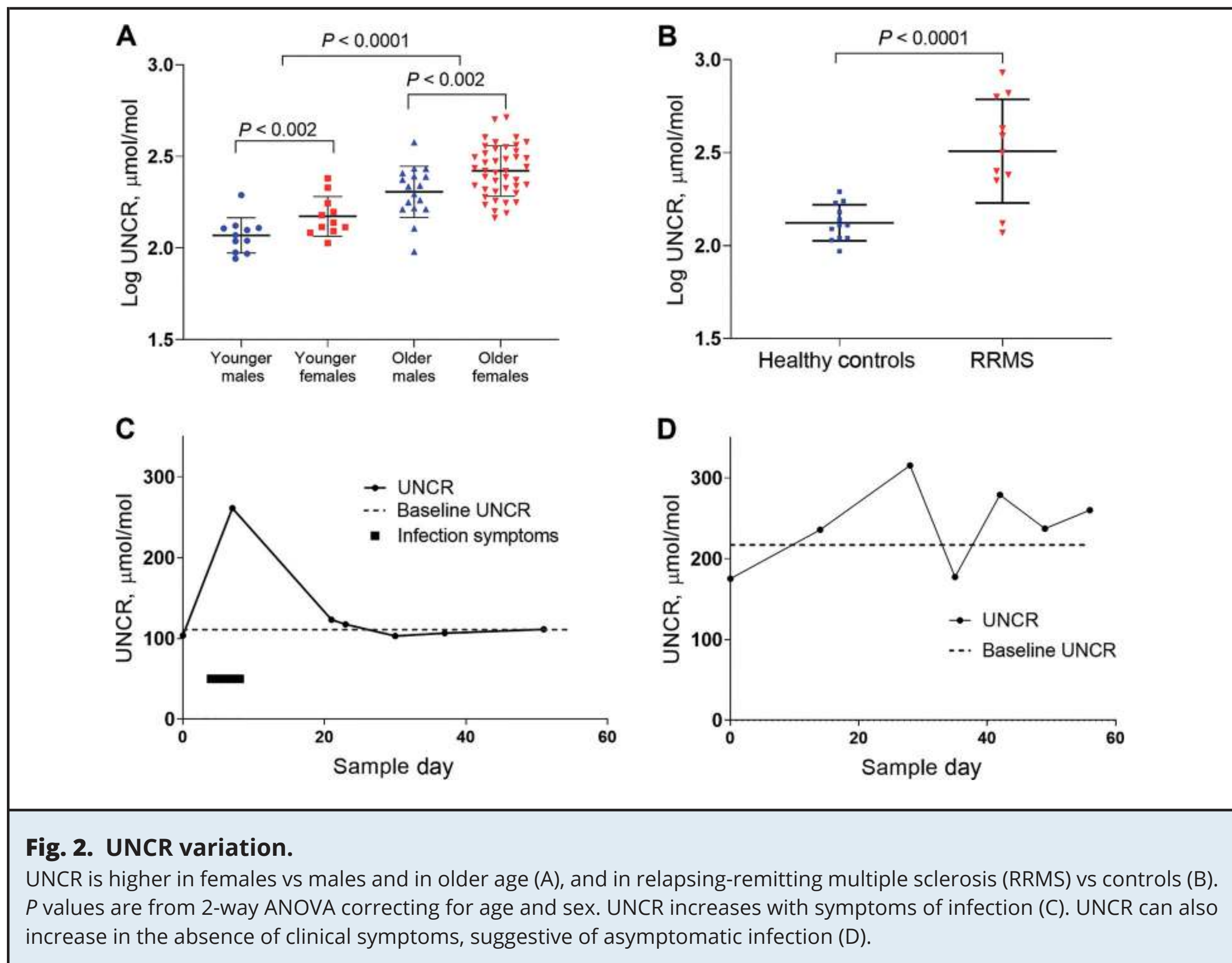

Fig. 2. UNCR variation.
UNCR is higher in females vs males and in older age (A), and in relapsing-remitting multiple sclerosis (RRMS) vs controls (B). *P* values are from 2-way ANOVA correcting for age and sex. UNCR increases with symptoms of infection (C). UNCR can also increase in the absence of clinical symptoms, suggestive of asymptomatic infection (D).

Spot UNCR. UNCR is reported to be higher with increasing age and in female compared with male individuals (*28*, *29*). To confirm this using our assay, spot urine samples were collected from older (n = 59; average age = 69.4 ± 3.2 years; male/female ratio = 17:42) and younger (n = 22; average age = 25.1 ± 5.8 years; male/female ratio = 11:11) healthy control individuals. UNCR was higher in the older age group and in female individuals (Fig. 2A). In a 2-way ANOVA of UNCR, both age and sex significantly influenced UNCR [age: $F(1,78) = 54.69$, $P < 0.0001$, partial $\eta^2 = 0.41$; sex: $F(1,78) = 12.97$, $P = 0.002$, partial $\eta^2 = 0.14$].

To confirm that UNCR as measured with our assay is sensitive to systemic inflammation, we compared spot urine samples from patients with relapsing-remitting multiple sclerosis (n = 11) and healthy controls (n = 12) (Fig. 2B; see also Table 2 in the online Data Supplement for demographics). In an analysis of covariance of UNCR controlling for sex and age, multiple sclerosis patients had significantly higher UNCR compared with healthy controls [$F(1,19) = 24.45$, $P < 0.0001$, partial $\eta^2 = 0.563$].

We then applied our method to study longitudinal fluctuations in UNCR, in relation to symptoms suggestive of an infectious or inflammatory event, in healthy individuals (n = 11; average age = 21.5 years; sex ratio, M/F = 7:4) who collected weekly urine samples during an 8-week period in autumn.

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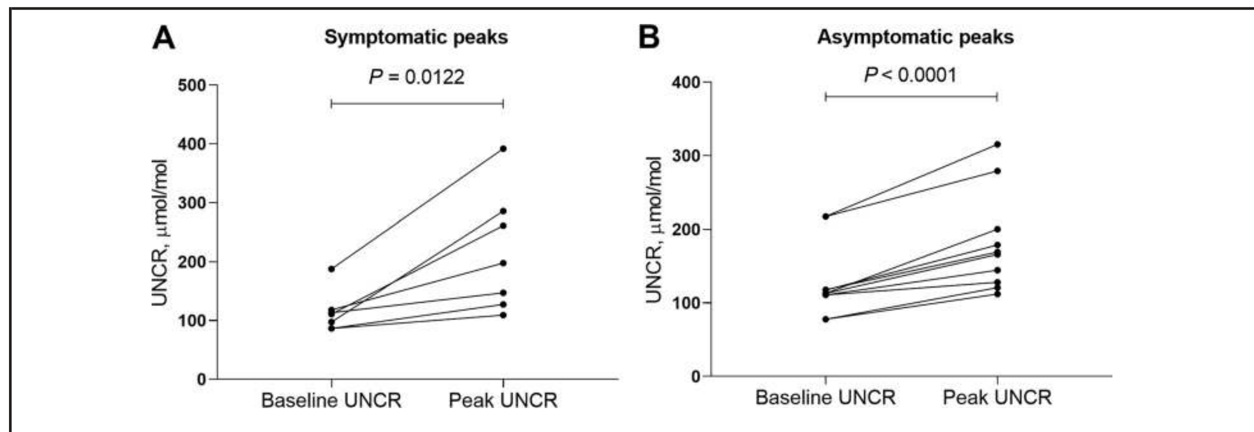

Fig. 3. Symptomatic and asymptomatic UNCR peaks.
Each person's individual baseline UNCR is paired with their respective peak UNCR during symptomatic (A) and asymptomatic (B) episodes. *P* values are from 1-tailed paired *t*-tests.

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Participants kept a daily record of any symptoms and medical events, such as infections, dental procedures, vaccinations, surgery, or injuries. Periods of raised UNCR (UNCR peaks) were identified using automated peak analysis in GraphPad Prism, with each individual's mean UNCR as baseline. Peak identification was also conducted visually, blinded to symptomatic events. Once peaks were identified, the baseline was adjusted to exclude peak UNCR values. When peaks were defined as an increase of 30% above the individual's baseline UNCR, there was 100% concordance between visual and automated methods of peak identification. A UNCR peak was considered to be associated with symptoms of an infection if it occurred within a week of reported symptoms. Six volunteers developed a total of 9 events with symptoms suggestive of infection that were associated with a UNCR peak (Fig. 2C and Fig. 3A) [$t(6) = 3.542$; $P = 0.0061$, 1-tailed paired *t*-test]. In 2 of these individuals, there were 2 symptomatic episodes close to each other, which were associated with the same broad peak. Hence, there were 7 UNCR peaks associated with symptomatic episodes. A toenail removal and a laceration in the leg were not associated with a UNCR peak. Additionally, 10 UNCR peaks in 6 individuals occurred in the absence of symptoms [$t(9) = 6.948$; $P < 0.0001$, 1-tailed paired *t*-test] (Fig. 2D and Fig. 3B). One of these individuals had received an influenza vaccine 3 days before the recorded UNCR peak. There was no difference in the percentage of UNCR increase above baseline between symptomatic and asymptomatic peaks [$t(9.106) = 1.516$; $P = 0.1633$, 2-tailed *t*-test with Welch correction; see Fig. 3 in the online Data Supplement]. Only 2 individuals in this cohort had troughs, defined in the same way as peaks, but below the baseline. One was not associated with any symptom or event. The other was associated with the influenza vaccination and followed the peak.

AUC UNCR. Serial sampling and calculation of the UNCR AUC normalized for follow-up time might provide a more accurate estimate of an individual's overall inflammatory status over a period because it is likely to be less affected by day-to-day fluctuations in UNCR. To investigate whether frequency of sampling makes a difference, we compared the UNCR AUC from monthly urine samples vs UNCR AUC from weekly urine samples in 20 progressive multiple sclerosis patients (see demographics in Table 3 of the online Data Supplement) who underwent weekly urine sampling over a 2.6-year

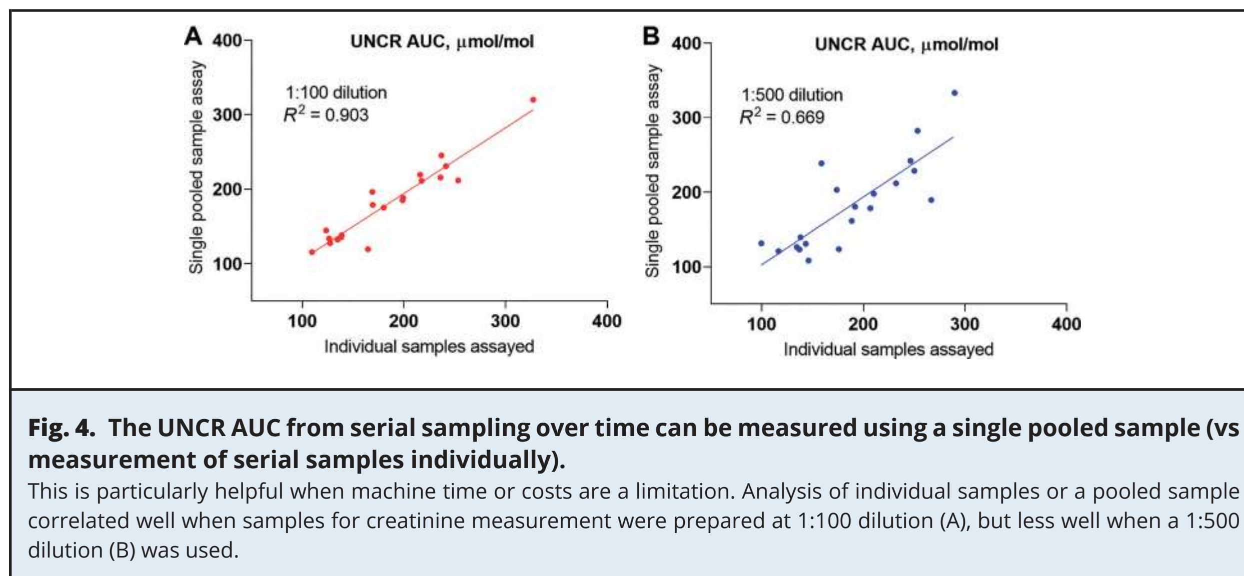

Fig. 4. The UNCR AUC from serial sampling over time can be measured using a single pooled sample (vs measurement of serial samples individually).
This is particularly helpful when machine time or costs are a limitation. Analysis of individual samples or a pooled sample correlated well when samples for creatinine measurement were prepared at 1:100 dilution (A), but less well when a 1:500 dilution (B) was used.

period. The monthly samples were selected using a random number generator. A paired *t*-test showed a significant difference between the UNCR AUC calculated from weekly and monthly values [$t(19) = 2.805$; $P = 0.0113$]. Although this may indicate that weekly urine sampling delivers more information than monthly sampling, the increased sampling frequency has cost implications. Therefore, we investigated whether analysis of a single sample derived from pooling weekly samples could replace AUC calculated from individual analysis of weekly samples to reduce running costs. Sample sets (n = 20) consisting of weekly urine samples taken over an average of 2.6 years were (*a*) measured individually and (*b*) pooled into one sample, which was then measured as a single sample. In clinical practice, the time intervals between serial samples may not be exactly the same, and there may be missing samples. Hence, the volume of serial samples cannot be pooled in equal volumes. To help with the preparation of the pooled sample, we developed a sample pooling calculator (SPoC) (available at https://eprints.soton.ac.uk/431818/) that calculates the volume of each sample to add to the pooled sample, based on the time interval between consecutive samples (see SPoC manual at https://eprints.soton.ac.uk/431818/ for methodology). When samples were prepared at 1:100 dilution, results from the 2 methods to measure UNCR AUC correlated well (R^2 = 0.903) but less well when a 1:500 dilution was used (R^2 = 0.669) (Fig. 4).

DISCUSSION

In chronic inflammatory diseases, systemic infections may increase the severity of symptoms or contribute to the overall progression of the disease (*30*). Hence, tracking systemic inflammation is important for research studies in this area. It also has significant potential utility in clinical management, when assessing response to treatment in inflammatory or infectious diseases. Urine sampling is noninvasive, so UNCR measurement provides a practical and simple means for clinical practitioners and researchers to track inflammation or response to treatment in inflammatory disorders. Patient cooperation is required to collect urine samples on certain days of the week using simple consumables supplied by the laboratory, date and/or time them, and store them in their

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home freezer. In our experience, even disabled patients with progressive multiple sclerosis find this to be easy and acceptable.

Here we have demonstrated a robust and sensitive analytic technique for systemic inflammation monitoring using UPLC-MS/MS, taking advantage of the neopterin biochemical pathway in activated myeloid cells. Similar methods have been previously reported (*23*, *31*, *32*). The method described here requires minimal sample preparation, enables high sample throughput, is cost-effective, possesses a low LOQ, and has excellent intraassay and interassay CV, linearity of dilution and spike and recovery while including both neopterin stereoisomers. Although 2 dilutions are used, neopterin and creatinine are measured in the same run. Because of this, the LC-MS method presented here is advantageous over a dual kit/platform approach as it is performed by the same technician using the same machine, within the same machine run, in the same laboratory. This avoids common errors associated with performing assays on different days (batch effects) or in different laboratories using different techniques. In all the clinical scenarios we tested, the changes in UNCR observed were above the assay's LOQ and the variations observed in experiments assessing assay precision and recovery after spiking.

L-threo-neopterin is a stereoisomer of D-erythro-neopterin that has so far received little attention. L-threo-neopterin is not always present, and the factors determining its production alongside D-erythro-neopterin need further study. In humans, L-threo-neopterin is synthesized by monocytes and macrophages in response to IFN-γ via the same biosynthetic pathway as D-erythro-neopterin (*33*). Hence, it is important to be able to include both isomers in peak area measurements because the reason they are measured is to act as a surrogate marker for the process (inflammation) that has generated them. We have shown in spike-and-recovery experiments that when L-threo-neopterin is present, it can be measured reliably using the D-erythro-neopterin standard curve.

Although measurement of 7,8-dihydroneopterin in urine, in addition to neopterin, is useful (*34*), it is unstable and difficult to reliably measure, making the quantification of downstream neopterin more practical in clinical practice or research studies embedded within busy clinical services. Indeed, it has been shown that measurement of neopterin has similar potential to the combined measurement of neopterin and 7,8-dihydroneopterin in clinical practice (*35*). The ratio of 7,8-dihydroneopterin to neopterin in body fluids is generally stable (3:1) (*36*).

In healthy controls, the absolute UNCR values using our assay are in agreement with those found in the literature. We also observed higher UNCR in the majority of older individuals and in female vs male individuals, as described before (*28*, *29*). Hence, UNCR needs age- and sex-specific reference ranges. As we, and others, accumulate more UNCR data over the next few years, these ranges will become available.

To demonstrate the clinical utility of the assay as a marker of systemic inflammation, we applied it to 2 settings. First, we found a higher UNCR in multiple sclerosis patients compared with healthy controls, as expected in an inflammatory condition (*14*, *37*). Second, weekly urine sampling in healthy controls identified UNCR peaks in association with periods of symptoms suggestive of infection. An interesting observation was that UNCR peaks also occurred in the absence of such symptoms. One such peak was associated with influenza vaccination, which suggests that asymptomatic UNCR peaks may represent episodes of immune activation caused by, for instance, subclinical infections or stochastic fluctuations in immune responses.

This substudy has several limitations; the infectious nature of the symptoms was not established serologically, and the characteristics of the participants (young and healthy) preclude generalizability. Further study is required to

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dissect the precise causes of asymptomatic UNCR peaks.

Healthy individuals had different baselines (CV, 32.6%). This underlines the importance of establishing a baseline for the individual when interpreting serial UNCR measurements. Hence, if one is asking whether in a particular individual there has been a UNCR increase in a longitudinal study, during which UNCR is measured serially over time, establishing a baseline for the individual would be necessary because of variability in the individual's baseline. How often should one sample? We have demonstrated how weekly and monthly urine sampling give different results; more frequent sampling is intuitively closer to the ground truth. However, a higher frequency of sampling is costly and time-consuming. We have shown that analysis of a single sample pooled from serial samples may suffice, provided that care is taken to ensure that the volume of serial sample added is proportional to the time interval between that serial sample and the next; we provide software for automated calculation of these volumes requiring minimum user input. Pooling of urine is done by the researcher, not by the patient, if analysis of a single sample is selected to be the method of choice to integrate the longitudinal UNCR profile over time. The pooling approach has limitations. High dilution during pooled sample preparation was less accurate probably because of the increased risk of pipetting errors. Also, pooling does not allow kinetic correlation between clinical events and UNCR.

In conclusion, UNCR is a useful biomarker of systemic inflammation. The UNCR assay method presented here has been comprehensively assessed, and offers simplicity, speed, robustness, reproducibility, and proven utility in clinical scenarios. UNCR fluctuations underline the importance of longitudinal monitoring, vs a single time point, to capture a more representative estimate of an individual's inflammatory state.

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