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Analytical Validation of GFR_{NMR}: A Blood-Based Multiple Biomarker Assay for Accurate Estimation of Glomerular Filtration Rate

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Abstract: Accurate and precise monitoring of kidney function is critical for a timely and reliable diagnosis of chronic kidney disease (CKD). The determination of kidney function usually involves the estimation of the glomerular filtration rate (eGFR). We recently reported the clinical performance of a new eGFR equation (GFR_{NMR}) based on the nuclear magnetic resonance (NMR) measurement of serum myo-inositol, valine, and creatinine, in addition to the immunoturbidometric quantification of serum cystatin C, age and sex. We now describe the analytical performance evaluation of GFR_{NMR} according to the Clinical and Laboratory Standards Institute guidelines. Within-laboratory coefficients of variation (CV%) of the GFR_{NMR} equation did not exceed 4.3%, with a maximum CV% for repeatability of 3.7%. Between-site reproducibility (three sites) demonstrated a maximum CV% of 5.9%. GFR_{NMR} stability was demonstrated for sera stored for up to 8 days at 2–10°C and for NMR samples stored for up to 10 days in the NMR device at 6 ± 2°C. Substance interference was limited to 4/40 (10.0%) of the investigated substances, resulting in an underestimated GFR_{NMR} (for glucose and metformin) or a loss of results (for naproxen and ribavirin) for concentrations twice as high as usual clinical doses. The analytical performances of $GFR_{NMR,}$ combined with its previously reported clinical performance, support the potential integration of this NMR method into clinical practice.

Keywords: glomerular filtration rate; eGFR; metabolite; NMR; analytical validation; linearity; precision; trueness; interference; stability

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1. Introduction

Chronic kidney disease (CKD) is a leading public health burden affecting more than 50 million people worldwide [1–3]. Kidney Disease: Improving Global Outcomes (KDIGO) defines CKD as abnormalities of kidney structure or function, present for >3 months, with negative health implications. CKD criteria include a decreased glomerular filtration rate (GFR < 60 mL/min/1.73 m^2) and/or 1 or more markers of kidney damage, such as albuminuria [4,5].

A Precise determination of GFR requires measurement of an exogenous tracer substance that is both freely filtered by the kidney and does not undergo metabolism, tubular secretion or absorption. As such, the gold standard for determining GFR is inulin clearance [2,6–9]. However, the use of such exogenous markers is impractical and costly. Therefore, endogenous GFR markers, such as serum creatinine and/or cystatin C, are measured to estimate GFR using validated eGFR equations in routine clinical practice [9–15].

Although in use for decades, eGFR equations present important limitations. These include the impact of body mass, diet, and age, which can influence metabolite production, tubular secretion, reabsorption, or extra-renal excretion [3,10,13,16,17]. Such limiting factors may result in significant deviations of eGFR from tracer-measured GFR (mGFR), incorrect CKD staging, and different rates of GFR decline [3,8,10,13,16,17], all of which limit the clinical utility of proposed eGFR equations.

In addition to biological limitations affecting clinical performance of eGFR, the analytical performance of metabolite measurements by a particular method can greatly impact the precision and accuracy of eGFR equations [8,18–21]. The factors potentially influencing the analytical performance of an eGFR assay include analytical precision and linearity of the measurement method, the impact of substances interfering with marker quantification in the sample, and sample stability [8,10,19,20,22–25]. Assay calibration has allowed for the reduction in errors associated with metabolite measurement, as well as reduce inter-laboratory variability of eGFR methods [3,18,21,26]. Altogether, analytical validation is essential for the standardization and implementation of new eGFR assays in clinical laboratories.

We have recently reported the clinical performance of a new blood-based assay, GFR_{NMR}, for the accurate estimation of GFR (normalized to 1.73 m^2 body surface area) in subjects with and without CKD [15]. The GFR_{NMR} equation is based on measurements of serum myo-inositol, valine, creatinine, and cystatin C, and integrates age and sex [15]. Practically, the GFR_{NMR} assay includes the simultaneous measurement of serum creatinine, valine and myo-inositol by nuclear magnetic resonance (NMR) spectroscopy, in addition to serum cystatin C measurement using a validated particle-enhanced turbidimetric immunoassay (PETIA) [27,28]. Clinical performance validation demonstrated a lower bias of the GFR_{NMR} equation to mGFR and a higher P15 accuracy compared to recommended eGFR equations (including Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI] and European Kidney Function Consortium [EKFC] equations) [15].

We now describe the analytical performance validation of the GFR_{NMR} assay, including both the analytical validation of the NMR measurements of serum creatinine, valine, and myo-inositol, and of the resulting GFR_{NMR} equation. We assessed metabolite measurements by NMR and GFR_{NMR} scores for repeatability and reproducibility, range of linearity, result stability in standard laboratory conditions, and the influence of potentially interfering substances. We show that metabolite measurement by NMR and GFR_{NMR} scores present analytical performances compatible with routine clinical practice, with high repeatability and reproducibility, a broad range of linearity, result stability, and limited influence of potentially interfering substances.

2. Materials and Methods

2.1. Blood Serum

Human serum was obtained from the Blood Donation Service of the Bavarian Red Cross as single-donor material prepared from 0.5 L of whole blood. The study used anonymized data (no personal reference can be established) and consequently was not subject to ethical consultation by the responsible ethics committee of the Bavarian Medical Association. All donating subjects gave written informed consent to use residual serum for research according to the Declaration of Helsinki (revised version of the 64th WMA General Assembly, Fortaleza, Brazil, October 2013), and following the standards of ICH-GCP E6 (R2).

2.2. Serum Cystatin C Measurements

Serum cystatin C measurements were performed with the Tina-Quant Cystatin C Gen.2 assay (Roche) on cobas 8000 Modular Analyzer (Roche) by an external accredited laboratory (Labor Staber, Munich, Germany), using the human serum certified reference material (CRM) ERM-DA471/IFCC (European Commission Joint Research Centre, Institute for Reference Materials and Measurements, Belgium) as cystatin C calibrator [29].

2.3. NMR Sample Preparation

Samples were prepared by mixing 540 µL serum with 60 µL of AXINON serum additives solution 2.0. A total volume of 600 µL was filled into a 5 mm NMR tube and capped with a barcoded cap for identification. Calibrator and quality control samples were prepared by filling 600 µL of AXINON serum calibrator or control 2.0, respectively, into a 5 mm NMR tube and capping with a barcoded cap for identification.

2.4. NMR Analysis and Biomarker Quantification

NMR analysis was performed as described elsewhere [30]. Prepared samples were pre-heated at 37 °C for 7.5 min before NMR measurement in a Bruker Avance III 600 MHz and a 5 mm PATXI probe equipped with automatic Z gradients shimming. A modified version of the CPMG pulse sequence was used. ^{1}H-NMR spectra were recorded using a spectral width of 20 ppm, with a recycling delay of 1.5 s, 16 scans, and a fixed receiver gain of 50.4. A cycling time d2 of 8 ms was used together with a corresponding T2 filter of 112 ms. Mixing time τ between 2 consecutive spin echoes was 400 µs. NMR data were automatically phase- and baseline-corrected using the lactate doublet at 1.32 ppm as reference. Metabolite quantification used curve-fitted pseudo-Voigt profiles [30]. In case of severe or frequent overlapping signals derived from interfering substances in the targeted spectral region, simultaneous fitting for the metabolite and the interfering substance(s) were performed [31].

2.5. Detection Capability (LoB, LoD, LoQ)

The determination of limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) for creatinine, valine and myo-inositol NMR measurements was performed according to the Clinical and Laboratory Standards Institute (CLSI) guideline EP17-A2 [32]. To generate blank fractions for LoB, aliquots of 4 different serum samples were cleared of small metabolites by dialysis against 1x phosphate buffered saline (PBS) supplemented with 10 mg/dL sodium-(L) Lactate, using Slida-A-Lyzer Mini Dialysis Devices with a molecular weight cutoff of 20,000 kDa (ThermoFisher Scientific, Waltham, MA, USA). A compilation of measurements from a number of samples, rather than a single sample, was generated as recommended [32]. For the determination of LoB, a total of 45 replicates for each of the 4 different dialyzed serum pools were measured within three days using three batches of reagents. The required low-level samples for LoD and LoQ determination were prepared by mixing normal serum samples with dialyzed serum samples in different levels until the desired concentration ranges were obtained. For the determination of LoD and LoQ, a total of 45 replicates for each of the different mixed low-level serum sample pools were measured within 3 days using three batches of reagents.

2.6. Linearity

Linearity of creatinine, valine and myo-inositol NMR measurements were determined according to CLSI guideline EP6-A [33]. To generate a low-level fraction, an aliquot of this serum was cleared of small metabolites by dialysis against 1× PBS supplemented with 10 mg/dL of sodium-(L) Lactate as described above. To generate a very high-level fraction, another aliquot of the serum was supplemented with creatinine (Sigma Aldrich, St. Louis, MO, USA), valine (Sigma Aldrich) and myo-inositol (Sigma Aldrich) to final concentrations >1 mmol/L for creatinine and valine, and >0.4 mmol/L for myo-inositol. A total of 11 equidistant concentration levels were prepared by linear intermixture of the high- and low-level fractions ranging from 100% high level to 0% low level, as recommended [33].

2.7. Precision

2.7.1. Single-Site Precision

Single-site precision was determined for the creatinine, valine, and myo-inositol analyte measurements and for the GFR_{NMR} equation. Repeatability, between-run precision, between-day precision and within-laboratory precision were calculated according to CLSI

guideline EP05-A3 [34]. Human serum from nine (analyte measurement precision) or 4 (GFR_{NMR} precision) donors was prepared in aliquots of 1.8 mL for a total of 20 experimental days and 2 runs per day. Aliquots were stored at −80 °C until use. The analyte measurement repeatability study was conducted over 20 days with 2 runs per day and 3 replicates per sample, using 9 serum sample batches chosen to cover low to high analyte concentrations (1080 measurements). The GFR_{NMR} repeatability study was conducted over 20 days with 2 runs per day and three replicates per sample, using 4 serum sample batches chosen to cover GFR_{NMR} values from impaired (below 60 mL/min/1.73 m^2) to physiological GFR around 90 mL/min/1.73 m^2 (480 measurements).

2.7.2. Multi-Site Precision

Multi-site precision was determined for the GFR_{NMR} equation. Repeatability, between-day precision, within-laboratory precision, and reproducibility were calculated according to CLSI guideline EP05-A3 [34]. Human serum from 4 donors was prepared in aliquots of 4.5 mL for 5 experimental days and one run per day on three different devices. Aliquots were stored at −80 °C until use. The reproducibility study was conducted on 3 devices at different laboratory sites, over 5 days, with 1 run per day, and 6 replicates per sample using 4 serum sample batches chosen to have GFR_{NMR} values covering the range from below 60 up to 90 mL/min/1.73 m^2 (360 measurements).

2.8. Method Comparison (Trueness)

Trueness of creatinine, valine and myo-inositol analyte measurements was determined using a reference method, when possible, or reference materials with known concentrations, according to the CLSI guidelines EP09-A3 and EP15-A3 [35,36].

2.8.1. Creatinine

A total of 150 serum samples were prepared as aliquots and a subset of 50 samples were analyzed at 3 independent sites. Additionally, creatinine reference measurements were performed with the Creatinine plus ver.2 (CREP2) enzymatic assay (Roche) on the legally marketed device cobas 8000 Modular Analyzer (Roche) by an external accredited laboratory (Labor Staber, Munich, Germany).

2.8.2. Valine

A total of 120 pooled human serum samples (4 mL each) were prepared from 2 to 3 single donor sera. A first set of 100 samples was measured without any modification. A second set of 10 samples was spiked with valine covering the high range of concentrations >1 mmol/L. A third set of 10 samples was dialyzed against 1× PBS supplemented with ≥10 mg/dL lactate, using Slida-A-Lyzer Mini Dialysis Devices with a molecular weight cutoff of 10,000 kDa (ThermoFisher Scientific). These samples were subsequently spiked with valine covering the lower concentration range from 0.2 to 0.4 mmol/L. Valine reference measurements were performed using the *AXINON*® lipoFIT assay (numares AG, Regensburg, Germany). Valine sample and reference sets were measured at a single site.

2.8.3. Myo-Inositol

In the absence of a valid reference method for myo-inositol measurement, trueness was evaluated by spike recovery experiments, as recommended in CLSI guidelines EP09-A3 and EP15-A3 [35,36]. A total of 150 human serum samples (4 mL each) were prepared from 2 to 3 single donor sera. For the lower concentration range, 44 of the pooled sera were dialyzed against 1× PBS supplemented with ≥10 mg/dL lactate, using Slida-A-Lyzer Mini Dialysis Devices with a molecular weight cutoff of 10,000 kDa (ThermoFisher Scientific, Waltham, MA, USA), which were then used for the desired low concentrations of myo-inositol. Each of the pooled human serum samples was divided into two aliquots. The first aliquot was used for mock-spiking with 5% volume of PBS to determine the mock levels of myo-inositol for the respective sample. The second set of aliquots was spiked with

5% volume of myo-inositol dissolved in PBS covering a concentration range from 0.02 to 0.40 mmol/L. Myo-inositol sample and reference sets were measured at a single site.

2.9. Sample Stability

Sample stability experiments were conducted to evaluate its impact on the GFR_{NMR} equation according to the CLSI guideline EP25-A [37].

2.9.1. Human Serum Specimen Storage Study (2–10 °C)

At baseline, blood was drawn from six donors using standard serum collection tubes with clotting activator without gel separator (Monovette-S neutral, Sarstedt, Nümbrecht, Germany). Samples were centrifuged within 2 h of collection at 1800× *g* for 10 min to separate serum from the clot. Immediately after centrifugation, separated serum was used for the baseline measurements. The remaining human serum was dispensed into aliquots of 2 mL for each donor. These aliquots were stored at 2–10 °C until they were prepared for analysis. For baseline measurements, five standard NMR samples were prepared immediately after centrifugation (t_0) and measured within the first six hours after collection. For all of the other time points (day 1 to 8), 1 aliquot was taken per donor and used for the preparation and measurement of three NMR replicate samples.

2.9.2. NMR Sample Storage Study (on Board)

For the storage study of NMR samples, the five NMR sample tubes prepared for the baseline measurement of the human serum storage study were stored in the sample changer of the device at 6 °C (±2 °C). The same NMR samples were repetitively measured at the selected time points (day 1, 4, 7, 9 and 10).

2.10. Interfering Substances

Interference testing was performed to evaluate its potential impact on the GFR_{NMR} equation according to CLSI guideline EP07 [38]. A total of 40 substances were identified to be clinically relevant and were tested for interference effects under 'worst case' conditions (Table S1). Per substance, 2 different serum pools with higher and lower GFR_{NMR} scores were prepared in 20 aliquots. Ten aliquots were spiked with the respective substance to the final concentration level as indicated in Table S1 and analyzed as test samples. The remaining ten aliquots were analyzed without spiked substance as control samples. Following this interference screen, a dose-response experiment was conducted for substances identified as potentially interfering with the GFR_{NMR}, using different A ("high") and B ("low") serum pools. A total of 5 equidistant concentration levels were prepared by linear intermixture of a 100%-spiked test pool and a non-spiked control pool to generate and measure five replicate fractions containing 0%, 25%, 50%, 75% and 100% of the potentially interfering substance.

2.11. Statistical Methods

All of the calculations of performance evaluation and statistical tests were performed within R 4.0.2 [39]. Data structures were handled with the data.table Package [40]. Visualization was performed with packages lattice [41] and ggplot2 [42]. In general, calculations followed the recommendations of the respective CLSI guidelines briefly described as follows.

2.11.1. Detection Capability

LoB, LoD and LoQ experimental data for creatinine, valine and myo-inositol were evaluated according to CLSI EP17-A2 [32]. LoB and LoD were determined depending on the distribution of the data for each parameter either by parametric or non-parametric method. For LoD calculation, the Cochran's C-test [43] was applied to check the assumption that the variability of measurement results is consistent across low level samples [32]. In case Cochran's *C* test failed, the "LoD Variant Approach: Nonparametric Analysis" was

used (trial and error experiment design according to EP17-A2). LoQ for each reagent lot and parameter was calculated as minimum mean concentration of the corresponding pools that showed a within-laboratory precision with a coefficient of variation (CV%) < 20%. The final overall LoQ was defined as the maximum LoQ over all individual lots.

2.11.2. Linearity

Linearity of creatinine, valine and myo-inositol NMR measurements was evaluated according to CLSI EP6-A [33]. The Pearson correlation coefficient [44] and repeatability (CV%) were calculated, and the maximum analytical concentration (upper limit of the linear range or LoL) was reported. Furthermore, polynomial regression analysis was performed for each analyte. For this, first-, second- and third-order polynomial models were built in the fashion of $y = b_0 + b_1x$, $y = b_0 + b_1x + b_2x^2$ and $y = b_0 + b_1x + b_2x^2 + b_3x^3$. A *t*-test was used to test whether non-linear coefficients (b_2 and b_3) were statistically significantly different (from 0). If no statistically significant terms were found ($p > 0.05$), then the data were considered as linear. If, however, a term was statistically significant ($p < 0.05$), then the degree of non-linearity was calculated for the best fitting polynomial according to standard error of regression. The linearity assessment for each analyte passed when a concentration range of at least five consecutive dilution levels had no more than 15% missing data, Pearson correlation coefficient *r* was ≥0.95, response appeared linear by visual inspection, repeatability (CV%) was <15%, and either no hint for non-linearity (no significant non-linear terms in 2nd or 3rd order polynomials) or a maximum relative degree of non-linearity <10% in case of detected non-linearity.

2.11.3. Single-Site Precision

Single-site precision was calculated for the creatinine, valine and myo-inositol analyte measurements, and for the GFR_{NMR} equation according to CLSI EP05-A3 [34]. All variance components (repeatability, between-run precision, between-day precision, and within-laboratory precision) were expressed as CV%. The acceptance criteria were that missing data per pool should not exceed 10% and that repeatability CV% should be ≤10% for GFR_{NMR}, ≤12% for creatinine, and ≤20% for valine and myo-inositol.

2.11.4. Multi-Site Precision

Multi-site precision was calculated for the GFR_{NMR} equation according to CLSI EP05-A3 [34]. All variance components (repeatability, between-day precision, within-laboratory precision, and reproducibility) were expressed as CV%. Only pools according to the use case (for example, pool mean GFR_{NMR} scores <90 mL/min/1.73 m^2) were analyzed. The acceptance criteria were that missing data per pool should be ≤10% and reproducibility CV% for GFR_{NMR} should be ≤10%.

2.11.5. Method Comparison (Trueness)

Trueness of creatinine, valine and myo-inositol analyte measurements was expressed as the bias between the test measurement (three different sites) and a reference value, as described in CLSI EP09-A3 and EP15-A3 [35,36]. Data were analyzed using Passing-Bablok regression [45,46]. The estimated regression equation by Passing-Bablok regression, and the Pearson correlation coefficient between observed and expected values, were reported. The acceptance criteria were a Pearson correlation coefficient $r \geq 0.90$ and a Passing-Bablok regression slope of 1.0 ± 0.15 (creatinine, myo-inositol) or 1.0 ± 0.075 (valine).

2.11.6. Sample Stability

Sample stability was evaluated according to CLSI EP25-A [37]. Stability duration was evaluated for each condition and donor at the level of the GFR_{NMR} equation. Based on the regression analysis, the one-sided upper or lower 95% confidence interval of the regression line was determined. In case of a positive and statistically significant ($p < 0.05$) regression slope, the intersection of the 1-sided 95% confidence interval upper limit and

the upper allowed drift limit defined the value of the stability duration. In case of a negative and statistically significant ($p < 0.05$) regression slope, the intersection of the 1-sided 95% confidence interval lower limit and the lower allowed drift limit defined the value of the stability duration. If the intersection was outside the defined period or if the regression slope was not statistically significant ($p \geq 0.05$), the value of the stability duration was set to the maximum time point experimentally tested. Furthermore, the overall stability duration for each condition was defined as the minimum of stability durations over all donors given a maximum allowable measurement drift of 10%. Acceptance criteria were that missing data should be ≤10% per condition, and that initial time point (t_0) as well as at least 2 additional time points having to be represented in the dataset.

2.11.7. Interference

Interference testing was evaluated according to CLSI EP07 [38]. Interference evaluation followed a two-step process, an interference screen followed by a dose-response experiment, if applicable. For the interference testing evaluation, missing data should not exceed 10% per comparison (by pool, substance, and parameter). If the mean difference in the results of spiked and non-spiked samples (mean GFR_{NMR} in test minus mean GFR_{NMR} in control, across each pool) was >10% of the mean GFR_{NMR} in control (defined as mean relative bias), an interference effect by the tested substance was anticipated. In such case, a dose-response experiment was conducted using new serum pools containing 0%, 25%, 50%, 75% and 100% of spiked substance. Relative bias (GFR_{NMR} in test samples vs. mean GFR_{NMR} in control) was calculated and represented as strip plot, to determine the concentration at which interference occurred (relative bias > 10% of control).

3. Results

The analytical performance of the metabolite measurements by NMR and of the GFR_{NMR} equation was evaluated according to the respective Clinical and Laboratory Standards Institute (CLSI) guidelines. Detection capability (LoB, LoD, LoQ) of creatinine, valine, and myo-inositol measurements are shown in Table 1. Linearity of the NMR measurements was demonstrated, with a Pearson correlation coefficient $r > 0.99$ for the three metabolites (Figure 1). The linear analytical range (LoQ–LoL) was 25–870 µmol/L for creatinine, 30–1255 µmol/L for valine and 39–439 µmol/L for myo-inositol (Table 1).

The trueness of the creatinine, valine, and myo-inositol NMR measurements was evaluated by comparison to a reference method (creatinine, valine) or by spike recovery (myo-inositol). The Passing-Bablok regression equations and the Pearson correlation coefficients (r) between observed and expected values are shown in Table 1. The Passing-Bablok regression slope was ≤1.05 and r was ≥0.99 for the three metabolites.

Single-site, within-laboratory precision for creatinine, valine, and myo-inositol NMR measurements was calculated from nine different serum pools covering a broad concentration range and a total of 1080 measurements. Within-laboratory coefficients of variation (CV%) did not exceed 12.5%, 2.2% and 16.5% for creatinine, valine, and myo-inositol measurements, respectively (Table 1). Within-laboratory CV% were lower in the higher concentration ranges > 3-fold LoQ (≤6.6%, ≤2.2% and ≤6.4% for creatinine, valine, and myo-inositol, respectively) (Table 1).

Table 1. Detection capability, upper limit of linear range, trueness and single-site precision evaluation of NMR measurements for creatinine, valine and myo-inositol.

	Creatinine		Valine		Myo-Inositol	
LoB [µmol/L]	20		20		39	
LoD [µmol/L]	25		26		39	
LoQ [µmol/L]	25		30		39	
LoL [µmol/L]	<870		<1255		<439	
Trueness PB; *r*	y = 1.028x−5.364; 0.990		y = 1.050x + 0.450; 0.996		y = 1.002x + 2.269; 0.990	
Precision [1]						
Pool	**Mean; SD [µmol/L]**	**CV%**	**Mean; SD [µmol/L]**	**CV%**	**Mean; SD [µmol/L]**	**CV%**
1	42.8; 5.4	12.5	211.4; 5.2	2.2	57.3; 4.4	14.6
2	62.8; 3.4	5.4	231.9; 5.4	2.1	63.4; 4.8	16.5
3	69.9; 5.4	7.8	280.2; 5.4	2.1	67.7; 5.2	14.3
4	76.0; 3.2	4.2	298.7; 4.6	2.0	69.1; 3.2	15.0
5	78.4; 5.2	6.6	315.3; 3.2	1.9	72.0; 3.4	15.8
6	85.4; 4.4	5.2	354.0; 3.4	2.1	80.4; 5.4	13.9
7	107.5; 4.8	4.5	383.5; 4.4	2.0	88.6; 4.6	11.7
8	123.8; 4.6	3.7	406.3; 5.9	2.2	204.6; 5.9	6.4
9	173.7; 5.9	3.4	463.8; 4.8	2.2	224.0; 5.4	5.9

[1] Single-site within-laboratory precision (1 site × 9 serum pools × 3 replicates per pool × 2 runs per day × 20 days; N = 1080 measurements). The nine serum pools were chosen to cover low to high physiological concentrations of the respective metabolites. Abbreviations: LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantification; LoL, upper limit of linear range; *r*, Pearson correlation coefficient; PB, Passing-Bablok regression; CV%, coefficient of variation (for within-laboratory precision) (%).

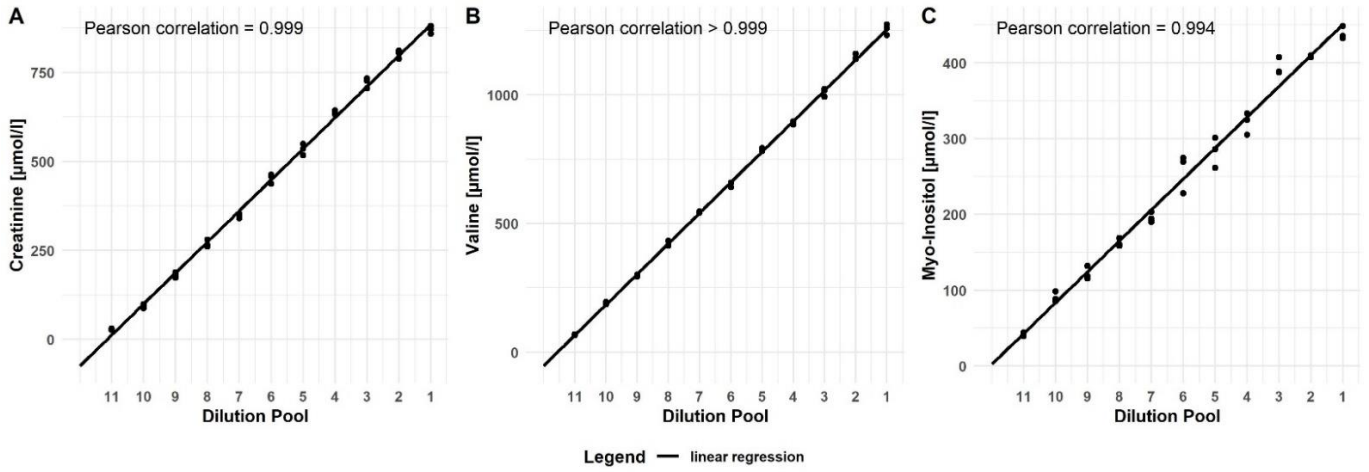

Figure 1. Linearity of NMR measurements for (**A**) creatinine, (**B**) valine, and (**C**) myo-inositol.

Single-site, within-laboratory CV% of the GFR_{NMR} equation, calculated from 4 different serum pools with GFR_{NMR} results ranging from 53 to 82 mL/min/1.73 m^2 and a total of 480 measurements, did not exceed 4.3%, with a maximum CV% for repeatability of 3.7% (Table 2). The inter-site reproducibility (between three sites), calculated from 4 serum pools ranging from 55 to 87 mL/min/1.73 m^2 and 360 measurements, resulted in a maximum CV% of 5.9% (Table 2 and Figure 2).

Table 2. GFR_{NMR} single- and multi-site precision.

Precision	N	Pool	Mean [mL/min/1.73 m^2]	Repeatability [CV%]	Between-Run [CV%]	Between-Day [CV%]	Within-Laboratory [CV%]	Reproducibility [CV%]
Single-site [1]	480	P1	53.5	2.8	1.3	2.4	3.9	n.a.
		P2	55.4	2.9	1.8	0.0	3.4	n.a.
		P3	78.1	3.7	0.0	1.2	3.9	n.a.
		P4	82.4	3.6	2.3	1.0	4.3	n.a.
Multi-site [2]	360	P1	55.4	3.3	n.a.	0.3	3.3	4.3
		P2	84.5	5.1	n.a.	2.8	5.8	5.9
		P3	84.7	2.9	n.a.	3.3	4.4	4.4
		P4	86.6	2.8	n.a.	2.1	3.5	4.0

[1] 1 site × 4 serum pools × 3 replicates per pool × 2 runs per day × 20 days (N = 480 measurements); [2] 3 sites × 4 serum pools × 6 replicates per pool × 1 run per day × 5 days (N = 360 measurements). Abbreviations: CV%, coefficient of variation; n.a., not applicable.

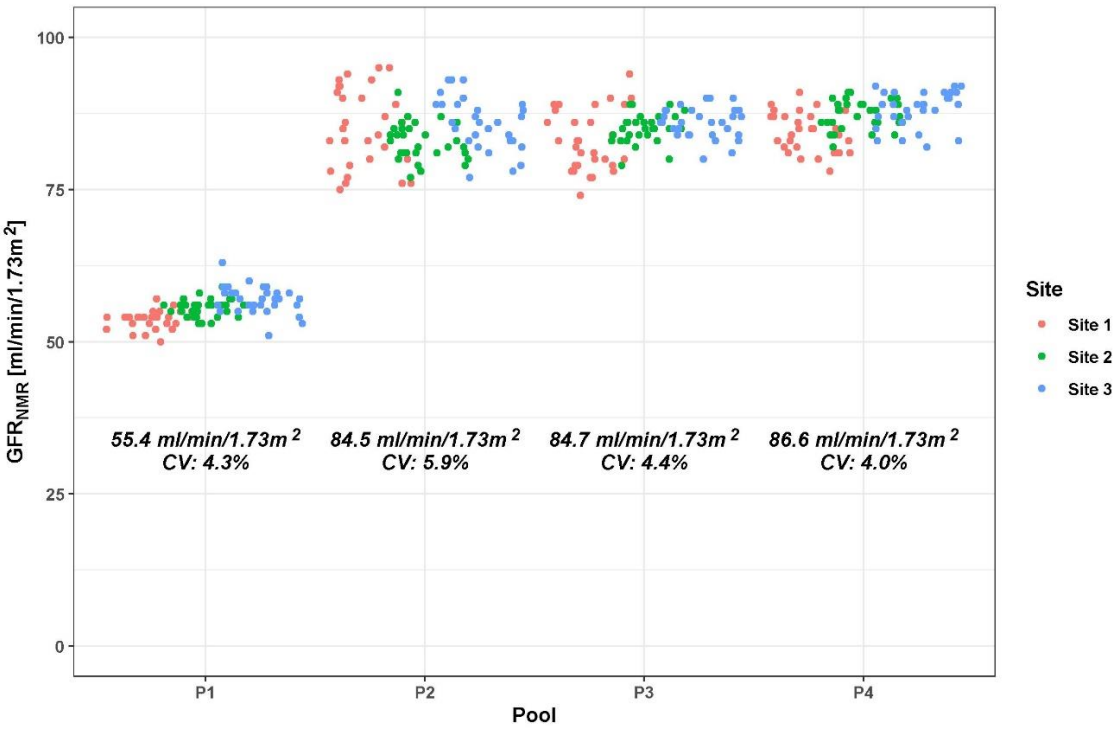

Figure 2. Scatterplot of multi-site precision (reproducibility) for the GFR_{NMR} equation calculated from 4 serum pools (P1–P4) with mean GFR_{NMR} scores below and above 60 mL/min/1.73 m^2. Abbreviation: CV%, coefficient of variation.

The influence of storage of serum samples and of prepared NMR samples on the stability of the GFR_{NMR} results was evaluated using individual donor samples and sample storage conditions reflecting those commonly used in clinical practice. GFR_{NMR} stability was demonstrated for serum stored up to eight days at 2–10 °C and for NMR samples stored up to ten days on board of the NMR device at 6 ± 2 °C (Table 3 and Figure 3). Over the storage time duration investigated, the linear regression slope *p*-values were not significant ($p > 0.05$), except for on-board NMR sample with a slope of 0.29 mL/min/1.73 m^2 per day, and a *p*-value of 0.043 (Table 3).

Table 3. GFR_{NMR} score stability during storage of human serum (2–10°C) and of prepared NMR samples (6 ± 2°C), relative to mean GFR_{NMR} at day 0 (t_0).

Stability	Donor	Mean GFR_{NMR} at t_0 [mL/min/1.73 m^2]	Slope [mL/min/1.73 m^2]	Intercept [mL/min/1.73 m^2]	Slope *p*-Value	Duration [days] [2]
Serum samples (2–10°C) [1]	1	105.6	−0.14	102.65	0.874	8
	2	78.0	−0.38	76.88	0.492	8
	3	109.8	−1.08	108.36	0.164	8
	5	106.6	−0.35	103.57	0.647	8
	6	99.6	0.21	97.03	0.723	8
On-board NMR samples (6 ± 2 °C)	1	105.6	−0.16	106.23	0.310	10
	2	78.0	0.08	77.42	0.327	10
	3	109.8	0.21	108.28	0.196	10
	4	118.0	0.29	117.77	0.043	10
	5	106.6	0.09	106.76	0.618	10
	6	99.6	0.03	99.26	0.865	10

[1] Volume of serum of Donor 4 was insufficient to cover all serum stability time points and was excluded from the analysis; [2] Calculated stability duration, as defined in Materials and Methods (2.11.6.).

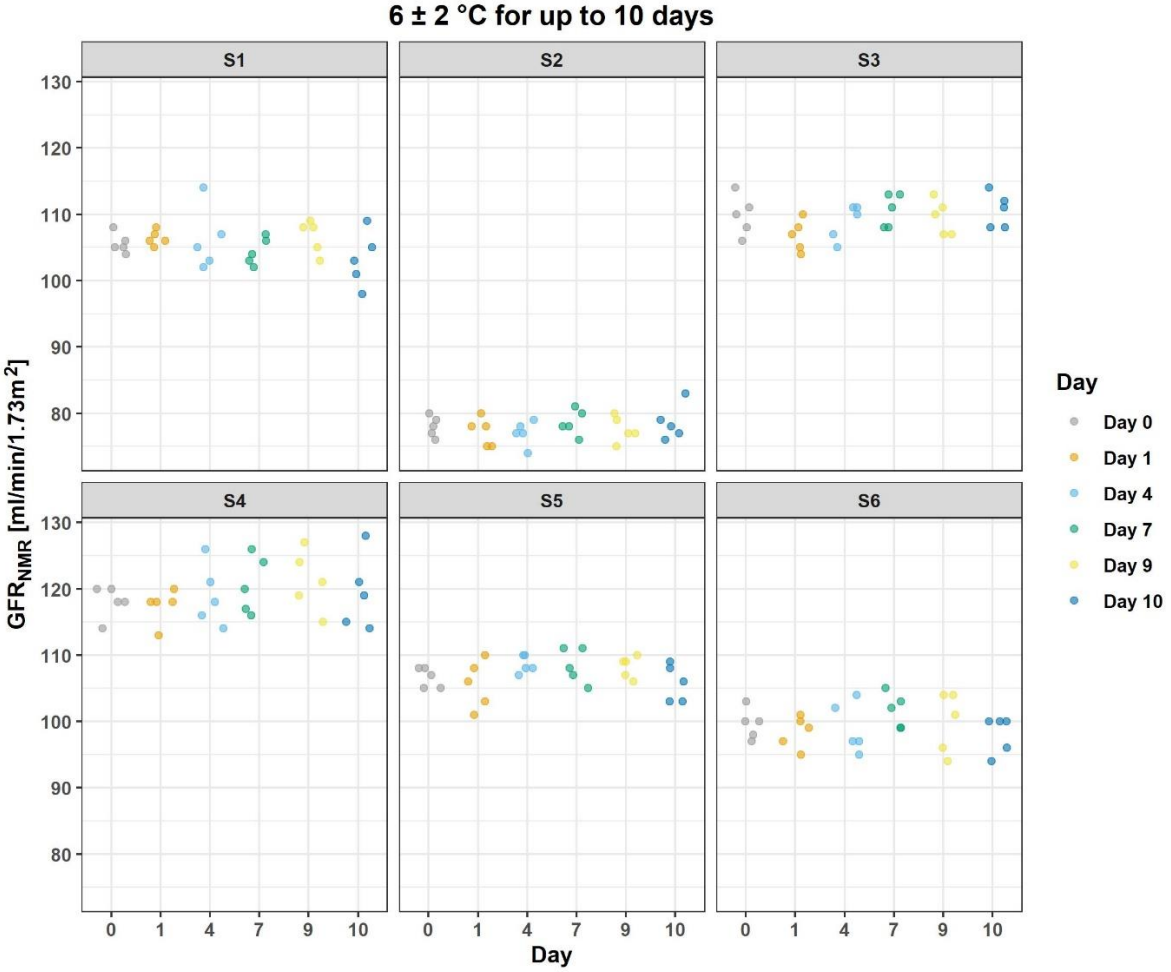

Figure 3. Scatterplot of GFR_{NMR} stability upon on-board storage at 6 ± 2°C of NMR samples for up to 10 days. The stability study was conducted using NMR samples prepared from the serum of six individual donors (S1–S6) and five replicate GFR_{NMR} measurements per time point.

Finally, we investigated a possible interference of the GFR_{NMR} assay by substances commonly expected to be present in clinical samples. Evaluated substances included endogenous metabolites indicative of clinically relevant metabolic disorders, such as ketone

bodies, common dietary constituents such as caffeine, antihistamines, over-the-counter drugs such as cetirizine or ibuprofen, antibiotics such as amoxicillin or gentamicin, major diuretics such as furosemide or chlorothiazide, cholesterol-lowering drugs such as atorvastatin, or antidiabetic drugs such as glipizide or pioglitazone (Table S1). We screened for relevant potentially interfering substances under 'worst-case' conditions, for example at the highest concentration expected to be observed in clinical settings. In case of prescribed drugs, these concentrations were determined at approximately three times their therapeutic daily dose. In case of endogenous metabolites, physiologically very high concentrations with a suitable safety margin were chosen (Table S1). A total of 40 potentially interfering substances were each spiked into serum pools with higher and lower GFR_{NMR} scores (Table S2). Of 80 spiked sera, 9 presented a mean relative bias to unspiked sera >10% and 2 presented no GFR_{NMR} results (Table S2 and Table 4). These 11 affected sera involved eight substances, namely glucose, ciprofloxacin, atorvastatin, metformin, naproxen, omeprazole, ranitidin and ribavirin (Table 4), which were further evaluated in dose-response experiments using independent serum pools (Figure 4).

Table 4. Potentially interfering substances with mean relative bias >± 10% in at least one serum pool in the interference screen.

Substance [1]	Pool [2]	Mean Relative Bias [%]
Atorvastatin	High	−12.20
Ciprofloxacin	High	+10.81
	Low	+11.84
Glucose	High	−23.34
	Low	−26.70
Metformin	High	−10.48
Naproxen	Low	−17.41
Omeprazole	High	−15.37
Ranitidin	High	−15.10
Ribavirin	High	No result
	Low	No result

[1] See Table S1 for tested concentrations; [2] Two serum pools with higher ("High") and lower ("Low") GFR_{NMR} values were tested per interference assay.

Dose-response experiments using concentrations of candidate interfering substances ranging from 25% to 100% of the concentration initially tested (Table S1) confirmed an interference for 4 substances: >13.9 mmol/L glucose, >23.2 µmol/L metformin, and >0.39 mmol/L naproxen caused falsely lower GFRNMR results, while >0.78 mmol/L naproxen and >210 mg/L ribavirin caused missing GFRNMR results (Figure 4). On the other hand, interference was not confirmed for atorvastatin, ciprofloxacin, omeprazole, and ranitidine (Figure 4). Thus, 4/40 (10.0%) tested substances showed some potentially interfering effect on the GFRNMR assay above certain serum levels.

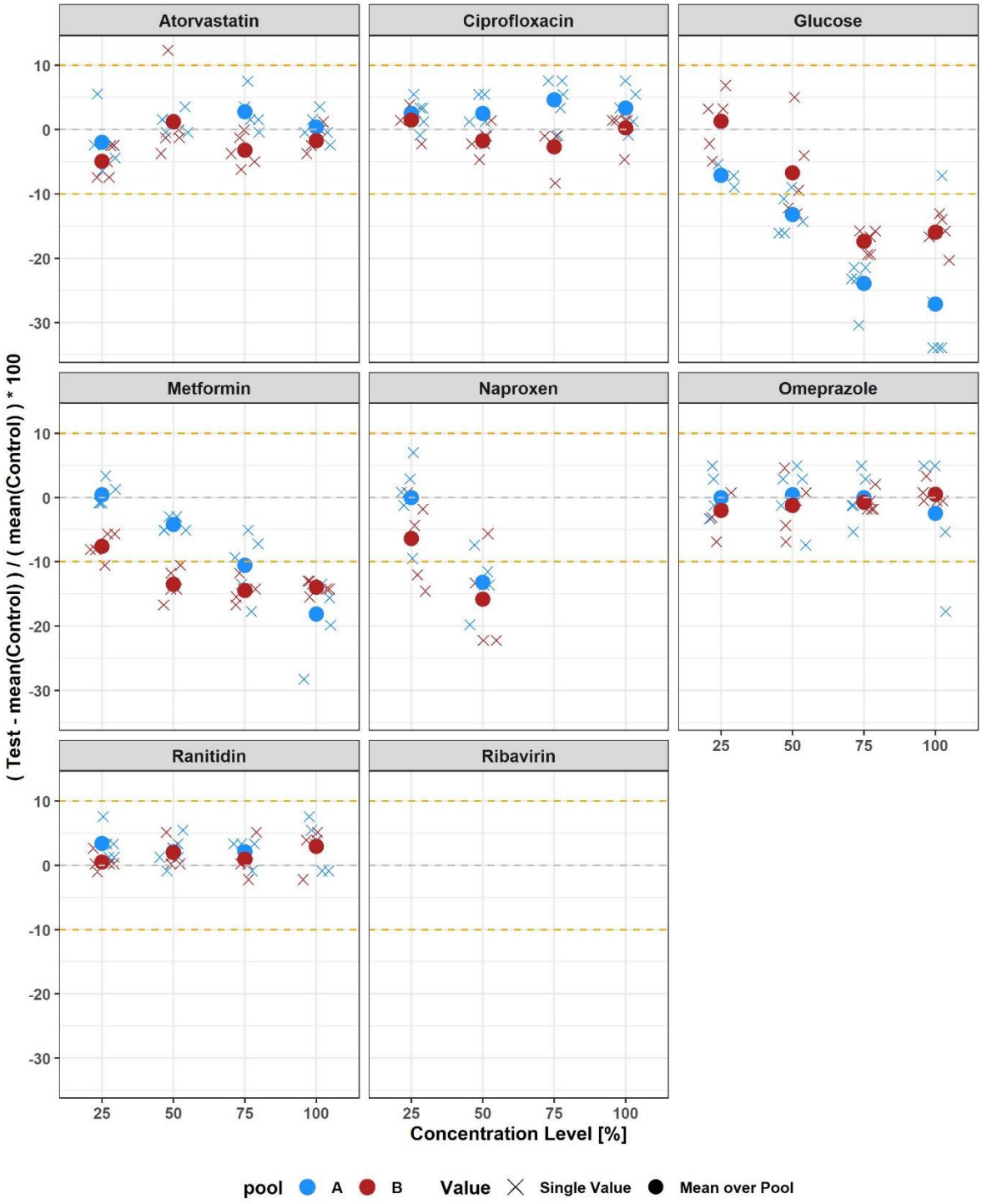

Figure 4. Dose−response interference experiments. Strip plots showing relative biases of GFR_{NMR} in serum pools (A: "low", B: "high", as defined in Table 4) spiked with increasing concentrations (0%, 25%, 50%, 75% and 100%) of the indicated potentially interfering substances (100% corresponding to the test concentration displayed in Table S1). Glucose > 13.9 mmol/L and metformin > 23.2 µmol/L caused falsely low GFR_{NMR} results, naproxen > 0.39 mmol/L caused falsely low or missing results at >0.78 mmol/L, and ribavirin ≥ 210 mg/L caused missing results. Interference suspected in the screening step (Table 4) was not confirmed in the dose-response assay for atorvastatin, ciprofloxacin, omeprazole and ranitidin.

4. Discussion

Laboratory testing for the evaluation of renal dysfunction includes estimation of glomerular filtration rate as the initial step. In this study, we describe the analytical performance validation of the standardized GFR_{NMR} assay, complementing its previously reported clinical validation [15]. We demonstrate GFR_{NMR} assay analytical performance as being compatible with its application in clinical routine settings, with linearity across a broad range of analyte concentrations, high precision, and comparability to reference methods, as well as stability $\geq$ 8 days under normal laboratory conditions. In direct comparison, detection capabilities of the NMR-based quantification of serum creatinine (LoQ of 25 µmol/L) were well below the 2.5%-tile of normal values of 45 µmol/L [47]. Imprecision of the creatinine quantification by NMR (CV% 3.4–12.5%) tended to be higher than that reported for conventional creatinine assays (CV% 0.4–4.4% for CREP2 Roche kit on Cobas c 503 based on IfU "2019-11, V 2.0 English"), but well below the 20% threshold recommended by the U.S. Food and Drug Administration. However, NMR technology allows the simultaneous detection of metabolite targets within a single analytical spectrum, resulting in a single level of impression for creatinine, valine, and myo-inositol as a biomarker constellation. As a direct consequence, the precision of GFR_{NMR}, including repeatability and reproducibility, was similar to that of other eGFR methods [20,22,24,25], despite the higher level of imprecision for the individual biomarkers. Moreover, the reported imprecision in gold-standard measures of GFR contributes to an appreciable proportion of cases in which mGFR can differ by $\geq$30% [48]. The analytical validation study reported here thus demonstrates that when samples are adequately collected and processed, excellent analytical precision and accuracy is anticipated in clinical practice.

No analytical interference was observed for the majority (36/40 [90%]) of the relevant substances tested. Interferences were detected in only 4/40 (10%) of the investigated agents, resulting in underestimated GFR_{NMR} (for >13.9 mmol/L glucose, >23.2 µmol/L metformin, and >0.39 mmol/L naproxen) or loss of results (for >0.78 mmol/L naproxen and >210 mg/L ribavirin). Our results suggest that the presence of substances, including additive solutions and therapeutic agents, will have little impact in clinical practice on the results of the GFR_{NMR} equation. However, the situation is different for patients with diabetes mellitus with serum glucose levels above 13.9 mmol/L (250 mg/L). Fasting plasma glucose levels >125 mg/dL (6.9 mmol/L) are considered indicative for diabetes [49]. Data from the National Health and Nutrition Examination Surveys 2005–2010 (NHANS) indicated fasting plasma glucose ranges of 89–177 mg/dL (4.94–9.82 mmol/L) in diabetic patients 20–44 years of age and 91–138 mg/dL (5.05–7.66 mmol/L) in patients $\geq$ 65 years of age [50]. Hence, in all cases, these values are well compatible with the observed interfering level of 13.9 mmol/L (250 mg/dL). Therefore, GFR_{NMR} testing after overnight fasting should be considered in diabetes management.

In addition, metformin is a glucose-lowering agent that is used as a first-line therapy for type 2 diabetes. Metformin is available in dosages of 500 mg, 850 mg and 1000 mg for oral administration to allow individualized blood glucose control. At usual clinical doses, metformin steady-state plasma concentrations are generally <1.5 µg/mL (11.6 µmol/L; [51]), for example less than 50% of the interference level obtained in our dose-response analysis. This makes a false-low GFR_{NMR} test result in vivo unlikely. Nevertheless, these results require further confirmation to verify, whether GFR_{NMR} underestimates GFR in patients with uncontrolled hyperglycemia on metformin treatment, as has been reported for Jaffé creatinine assays [52].

Naproxen >0.39 mmol/L interfered with GFR_{NMR}. Naproxen is a common analgesic, antipyretic and anti-inflammatory drug, which inhibits the formation of prostaglandins by inhibiting hormone-sensitive lipase [53]. In our experiments, other common analgetic drugs such as ibuprofen, acetylsalicylic acid, and acetaminophen did not interfere with GFR_{NMR} testing. In clinical practice, they could therefore be substitutes to naproxen when GFR_{NMR} is planned. Alternatively, GFR_{NMR} testing could be delayed after withdrawal in patients acutely treated with naproxen to avoid false-low or missing results.

In patients with or without CKD receiving active ribavirin treatment for hepatitis C, GFR_{NMR} should be replaced by alternative test methods to minimize the risk of missing test results with GFR_{NMR}.

Our study presents minimal limitations. First, we used samples from healthy donors only for the analytical validation analysis, thus not reflecting the lower range of eGFR that would be expected from patients with progressed CKD. However, we generated samples simulating conditions of patients with low eGFR, as recommended by CLSI. The expansion of the interference experiments by evaluating in-vivo samples of patients with a high likelihood for the presence of certain interfering substances, as shown by others [23], would have complemented our approach. This limitation was however compensated by the analysis of 'worst-case' scenarios and the analysis of a high number of substances, optimizing the probability of identifying putative interfering substances. Finally, in this analytical performance study, the impact of the cystatin C measuring method (Tina-Quant Cystatin C Gen.2 assay, Roche) was not considered for the analytical performance evaluation of GFR_{NMR}, which might introduce a bias in the evaluation. However, this is unlikely' given that the cystatin C assay was conducted with the recommended ERM-DA471/IFCC calibrator [29,54], and that its excellent analytical performance is well documented [27,28,54,55].

A strength of our analytical validation study is that it closely followed the recommendations of the CLSI guidelines, and that samples were stored and handled uniformly across the study. Moreover, our interference study included a large number (n = 40) of relevant potentially interfering agents, which contrasts with most reported analytical validation studies [20,23,24,56].

5. Conclusions

Developing a robust assay with fully characterized analytical properties is a critical step toward the implementation of a reliable and accurate biomarker-based diagnostic GFR assay into routine clinical practice. Here, we demonstrate an excellent analytical performance of the GFR_{NMR} assay, complementing its recently reported superior clinical performance compared to existing eGFR equations [15]. In regards to routine clinical practice, we implemented the GFR_{NMR} assay on an NMR platform without the need for pre-separation of analytes before quantification. Such an optimized process allows expedited sample processing with limited opportunities for human operator error, all of which make the GFR_{NMR} assay an attractive option for a reliable diagnostic test that can be run in a decentralized manner.

6. Patents

ES has a patent application WO002020065092A1 pending.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/diagnostics12051120/s1, Table S1: Substances selected for interference study with information about supplier, substance batch, final test concentration and comments on the substance clinical background; Table S2: Interference study results for substances described in Table S1, showing the mean GFR_{NMR} values for control and test pools and the relative bias for serum pools with higher and lower GFR_{NMR} scores.

Author Contributions: Conceptualization, M.F. (Markus Fuhrmann) and E.S.; methodology, J.V., V.R., A.S.S. and F.S.; validation, R.S., J.W.M., M.F. (Marianna Fernandes) and A.S.S.; formal analysis, J.V., V.R. and F.S.; investigation, M.F. (Markus Fuhrmann), F.S. and E.S.; resources, M.F. (Markus Fuhrmann), R.S., J.W.M., M.F. (Marianna Fernandes), A.S.S. and E.S.; data curation, J.V., V.R. and F.S.; writing—review and editing, M.F. (Markus Fuhrmann), E.S., J.V., V.R., A.S.S., F.S., R.S., J.W.M., M.F. (Marianna Fernandes) and J.E.; visualization, F.S.; supervision, M.F. (Markus Fuhrmann) and E.S.; project administration, E.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and following the standards of ICH-GCP E6 (R2). Ethical review and approval were waived for this study due to the use of anonymized leftover samples of healthy donors provided by the Bavarian Red Cross (03. November 2021).

Informed Consent Statement: Informed consent was obtained from all donors involved in the study.

Data Availability Statement: The data presented in this study are available within the article or Supplementary Materials.

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Conflicts of Interest: M.F. (Markus Fuhrmann), A.S.S., J.V., V.R., F.S. and E.S. report personal fees from numares AG, outside the submitted work. numares AG is a diagnostics company and focuses on the discovery, development and commercialization of diagnostic tests by metabolite constellations. J.E. serves as a scientific advisor for numares AG and receives financial compensation. M.F. (Marianna Fernandes) reports personal fees from Boston Heart Diagnostics. Boston Heart is focused on the treatment and prevention of disease by offering novel diagnostics that drive a personalized approach to improve patient health. All other authors have declared that no conflict of interest exists.

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