Sylvia A.A.M. Genet, Sebastian A.H. van den Wildenberg, Maarten A.C. Broeren, Joost L.J. van Dongen, Luc Brunsveld, Volkher Scharnhorst and Daan van de Kerkhof*

Quantification of the lung cancer tumor marker CYFRA 21-1 using protein precipitation, immunoaffinity bottom-up LC-MS/MS

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Abstract

Objectives: Numerous studies have proven the potential of cytokeratin 19 fragment 21-1 (CYFRA 21-1) detection in the (early) diagnosis and treatment monitoring of non-small cell lung cancer (NSCLC). Conventional immunoassays for CYFRA 21-1 quantification are however prone to interferences and lack diagnostic sensitivity and standardization. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an emerging approach based on a different, often superior, detection principle, which may improve the clinical applicability of CYFRA 21-1 in cancer diagnostics. Therefore, we developed and validated a protein precipitation, immunoaffinity (IA) LC-MS/MS assay for quantitative analysis of serum CYFRA 21-1.

Methods: Selective sample preparation was performed using ammonium sulfate (AS) precipitation, IA purification, tryptic digestion and LC-MS/MS quantification using a signature peptide and isotopically labeled internal standard. The workflow was optimized and validated according to EMA guidelines and results were compared to a conventional immunoassay.

Results: Significant interference effects were seen during IA purification, which were sufficiently solved by performing AS precipitation prior to IA purification. A linear calibration curve was obtained in the range of 1.0–100 ng/mL ($R^2=0.98$). Accuracy and precision were well within acceptance criteria. In sera of patients suspected of lung cancer, the method showed good correlation with the immunoassay.

Conclusions: A robust AS precipitation-IA LC-MS/MS assay for the quantification of serum CYFRA 21-1 was developed. With this assay, the clinically added value of LC-MS/MS-based detection over immunoassays can be further explored.

Keywords: cytokeratin 19 fragment 21-1; lung cancer diagnostics; protein precipitation; immunoaffinity (IA); tryptic digestion; liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Introduction

Cytokeratin 19 (CK19) is an intermediate filament protein found in simple epithelium including bronchial epithelium and tumors derived thereof [1–3]. During tumor cell degeneration, increased proteolytic activity induces cleavage of CK19 into soluble fragments that are released into the bloodstream and can serve as a marker for tumor growth. An example is cytokeratin 19 fragment 21-1 (CYFRA 21-1), which is the C-terminal part of CK19 that is assumed to be formed after cleavage by caspase 3 (Figure 1) [4–6]. Several studies have proven the potential of CYFRA 21-1 detection in the (early) diagnosis, prognosis and treatment monitoring of non-small-cell lung cancer (NSCLC) [7–11]. Combined assessment with other serum tumor markers (TMs) such as neuron-specific enolase (NSE), carcinoembryonic antigen (CEA) and progastrin-releasing peptide (proGRP) was
described to provide an increased diagnostic performance and aided in the differentiation of lung cancer subtypes [12–14]. Compared to the current gold standard based on imaging procedures and tissue biopsies, this less invasive TM approach offers great potential towards a fast and more patient friendly diagnostic trajectory.

Nowadays, CYFRA 21-1 is quantified using sandwich immunoassays such as radioimmunoassays, enzyme-linked immunosorbent assays, or electrochemiluminescence assays (ECLIA), all based on two selective antibodies against the C-terminal part of CK19, K191.1 (amino acids 311–335) and BM19.21 (amino acids 346–367) [7, 15]. However, clinical applicability of these assays remains limited as they are prone to interferences and lack standardization and diagnostic sensitivity (47–60%), which leads to a large group of missed NSCLC diagnoses [10, 12, 16]. A promising alternative method using a free-solution assay coupled to a compensated interferometric reader showed that this different detection principle can lead to significantly improved diagnostic performance [17]. Unfortunately, this assay format is not commercially available and clinical validation has not been published thus far.

In recent years, immunoaffinity (IA) liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays have been increasingly developed for the quantification of selected lung cancer protein TMs such as NSE and proGRP [18–21]. The MS/MS approach poses multiple advantages compared to immunoassays such as superior selectivity towards isoforms and post-translational modifications, which may offer new insights and improved identification of different types of lung cancer. It also allows LC-MS/MS-based assays to serve as a reference method for standardization of conventional immunoassays, which is an ongoing challenge in clinical laboratories. Moreover, the use of LC-MS/MS enables a multiplex platform to quantify multiple TMs in a single run. To enable clinical decision making based on serum analysis, IA LC-MS/MS assays should be carefully developed and validated to prevent interference- and matrix effects [22, 23].

In this study, a selective CYFRA 21-1 quantification method was developed by using two sample purification steps, ammonium sulfate (AS) precipitation and IA purification, and bottom-up LC-MS/MS analysis. The importance of using two purification steps to minimize interference- and matrix effects was shown. The assay was successfully optimized, validated, applied to patient samples and compared to results of ECLIA. This new CYFRA 21-1 quantification method could provide novel applications and insights in lung cancer diagnostics.

**Materials and methods**

**Chemicals and reagents**

Human CYFRA 21-1 calibrator grade, containing 0.083 mg/mL CYFRA 21-1 and a total protein concentration of 33.9 OD280 units, was purchased from Meridian Life Science (Memphis, TN, USA). Monoclonal anti-cytokeratin 19 antibody (A53-B/A2) produced in mouse was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Stable isotopically labeled internal standard (IS) AALEDTLAETAR-[13C6, 15N4] was obtained from Pepscan Presto BV (Lelystad, The Netherlands), dissolved in 10% acetonitrile (ACN), 0.1% formic acid (FA) and MilliQ at 900 ng/mL and stored in aliquots at −70°C. Protein G (pG) Dynabeads and BS(PEG)5 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cetuximab (Erbitux, Merck, Darmstadt, Germany) was buffer-exchanged to PBS pH 7.6 using an Amicon 50 kDa MWCO centrifugal filter (Merck Millipore) according to manufacturer’s instructions and the final protein concentration was checked (9.25 g/L). Adult bovine serum (BOV), purchased male AB serum (HUM-S) and all other reagents were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

**Antibody coupling to pG functionalized magnetic beads**

The coupling procedure of the K191.1 antibody to pG functionalized magnetic beads was performed multiple times in LoBind Eppendorf/Falcon tubes according to the following general procedure: 0.5 mg pG Dynabeads were washed twice in 400 μL conjugation buffer (100 mM sodium phosphate, 70 mM NaCl, 0.05% Tween-20 (v/v), pH 8.0). The supernatant was removed and 4 μg K191.1 antibody dissolved in conjugation buffer was added to the beads. After 30 min incubation with rotation, 25 μM BS(PEG)5 in cold conjugation buffer was added and incubated for 30 min with rotation. The reaction was quenched by adding 50 μL of 1 M Tris-HCl, pH 8.0 for 15 min with rotation.

**Figure 1:** CYFRA 21-1 sequence as part of CK19, formed after cleavage by caspase 3. The antibody binding regions of K191.1 and BM19.21 are indicated. The underlined peptide AALEDTLAETAR was chosen as SP, as it was shown to be unique in the human genome (italic), had no unfavorable amino acids (green), and was detected in IA QTOF LC-MS (bold). CYFRA 21-1, cytokeratin 19 fragment 21-1; CK19, cytokeratin 19; SP, signature peptide; IA QTOF LC-MS, immunoaffinity quadrupole time-of-flight liquid chromatography-mass spectrometry.
supernatant was removed and unbound antibody was eluted by adding 50 µL (elution 1) and 400 µL (elution 2) elution buffer (1 M NaSCN in 100 mM Tris-HCl, pH 7.5) for 5 min with rotation. The beads were then washed twice with 400 µL PBS, 0.1 % Tween-20, pH 7.6 and subsequently stored in this buffer at 4 °C until further use. For large-scale experiments, volumes were multiplied by the number of equivalents.

AS precipitation and IA purification of CYFRA 21-1

Serum samples were processed by subsequent protein precipitation and isolation of CYFRA 21-1 by IA purification. Protein precipitation was performed by slowly adding 1 mL of 4.1 mol/L AS to 1 mL serum in a 2 mL LoBind Eppendorf tube (50 % v/v AS). After 30 min incubation with rotation, the sample was centrifuged for 10 min at 13,400 rpm. The supernatant was discarded and the pellet was redissolved in 1 mL PBS, pH 7.6. IA purification was performed by adding 50 µL of the coupled beads to the dissolved pellet, which was then incubated for 1 h with rotation. Hereafter, the supernatant was removed and the beads were washed twice with 200 µL PBS, pH 7.6.

Tryptic digestion

The washed beads were shortly centrifuged to allow removal of all supernatant whereafter 33.1 µL digestion solution was added (31.5 µL 50 mM Tris-HCl, 1 mM CaCl₂, + 1 µL 1 M Tris-HCl, pH 8.0 + 0.2 µL IS per sample). The sample was denatured for 5 min at 95 °C at 1,200 rpm after which the mixture was cooled down and shortly centrifuged. Digestion was initiated by adding 300 ng trypsin for 1 h at 37 °C. The tryptic peptides of CYFRA 21-1 were evaluated based on in silico signatures, number of amino acids (6<n<25), presence of the unstable amino acids (M, C, N, Q) and signal intensity in mass spectrometry. The tryptic peptides of CYFRA 21-1 were isolated by IA purification, followed by denaturation, digestion, and consecutive high resolution quadrupole time-of-flight (QTOF) LC-MS/MS (Waters, Milford, MA) analysis to confirm sequence identity of the precursor and product ions. This data acquisition mode alternates low-energy and high-energy collision energies (CE), enabling the measurement of precursor and product-ions within one chromatographic run.

Signature peptide selection

The tryptic peptides of in silico digested CYFRA 21-1 were evaluated based on uniqueness in the human genome using BlastP software (https://blast.ncbi.nlm.nih.gov/Blast.cgi), number of amino acids (6<n<25), presence of the unstable amino acids (M, C, N, Q) and signal intensity in mass spectrometry. Since the CYFRA 21-1 stock also contained other proteins, 10 µg CYFRA 21-1 was isolated by IA purification, followed by denaturation, digestion, and consecutive high resolution quadrupole time-of-flight (QTOF) LC-MS/MS (Waters, Milford, MA) analysis to confirm sequence identity of the precursor and product ions. This data acquisition mode alternates low-energy and high-energy collision energies (CE), enabling the measurement of precursor and product-ions within one chromatographic run.

Instrumentation and chromatographic conditions

Signature peptide (SP) selection was performed in LC-MS³ mode on a Xevo G2 QTOF coupled to an Acquity UPLC I class binary solvent manager and Acquity UPLC Sample Manager-FL (Waters, Milford, MA, USA). Chromatographic separation was performed on an Agilent Polaris C18-A (2.0 × 150 mm, 3 µm) column with a flowrate of 0.3 mL/min and column temperature of 50 °C. The mobile phases consisted of (A) 0.1 % FA in MilliQ and (B) 0.1 % FA in ACN. The gradient in volume percentage of mobile phase B per minute was 5–60 % B (0.0–53.5 min), 60–80 % B (53.5–53.6 min), 80 % B (53.6–55 min), 80–5 % B (55–55.1 min) and 5 % B (55.1–60 min). Electrospray ionization (ESI) was operated in positive mode. Mass spectra were collected in MS³ mode with ramped CE from 20 to 30 V over the range of 100–2000 m/z. Peptide sequencing was performed using the BioLyx application within the software (Mas-sLyx 4.1, Waters, Milford, MA). Quantitative analysis was done on a LCMS-8045 triple quadrupole coupled to a Nexera-1 LC-2040C 3D Plus (Shimadzu, Kyoto, Japan). Chromatographic separation was carried out using the same column, flow rate and column temperature as described above with a gradient in volume percentage of mobile phase B per minute of 5–60 % B (0.0–13.5 min), 60–80 % B (13.5–13.6 min), 80 % B (13.6–15.0 min), 80–5 % B (15.0–15.1 min) and 5 % B (15.1–20 min). The ESI was operated in positive mode with the optimized transition settings of the SP and IS listed in Supplementary Table 1.

Assay calibration and validation

Calibration standards were prepared in BOV using 1.0, 1.6, 3.1, 6.3, 13, 25, 50 and 100 ng/mL CYFRA 21-1 and subsequently stored at −70 °C. For each analytical run, peak area ratios of the SP and IS were plotted vs. the nominal concentrations of the calibration standards using linear least-squares regression with a weighing factor of 1/x². The lower limit of quantification (LLoQ) was checked by comparing the peak area of the 1.0 ng/mL calibration standard with blank BOV samples (both n=4) at the retention time of the SP (LLoQ at least 10-fold higher compared to the blank). Quality control (QC) samples were prepared in BOV using CYFRA 21-1 concentrations of 1 ng/mL (LLoQ), 3 ng/mL (low), 40 ng/mL (medium) and 80 ng/mL (high). The within run and between run precision and overall accuracy were evaluated at the four QC levels by measuring five replicates over 3 days and analyzed using one-way analysis of variance according to CLSI protocol EP15-A2 [24]. Precision was expressed as coefficient of variation (CV, %) and the accuracy was expressed as percent bias (% bias). The results were compared to European Medicines Agency (EMA) guidelines, which recommend acceptance criteria for chromatographic methods of <15 %, with exception of the LLoQ, which should be <20 %.

Collection of patient serum

Sera were selected from a clinical study (lung marker study NL9146, ICTRP Search Portal [who.int]) described elsewhere [11, 14, 20, 25, 26], ethically approved by the Medical Research Ethics Committees United (NL58985.100.16). Here, whole blood was collected in an 8.5 mL BD Vacutainer SSTII Advance Plus Blood Collection Tube during venipuncture. The tubes were processed within 1 h after collection by centrifugation for 10 min at 2,683 g at 20 °C. Four patient serum pools (HUM-P) used to study the interference effects and matrix effects were made by combining residual serum samples of different patients in such a way that individual patients were only used for a single pool. Serum was stored in aliquots at −80 °C before analysis.

Quantification of human albumin, immunoglobulin, total protein and CYFRA 21-1

For the quantification of albumin and total protein, colorimetric assays were used. Immunoglobulin (IgG) was quantified using an
immunoturbidimetric assay. A commercially available ECLIA was used for the comparative quantification of CYFRA 21-1 (Roche Diagnostics, Rotkreuz, Switzerland). All assays were validated and performed on a Cobas platform (e801, Roche Diagnostics). The endogenous CYFRA 21-1 concentration in HUM-S was determined to be 1.3 ng/mL and the concentrations in HUM-P were 2.5, 3.4, 1.3 and 8.7 ng/mL.

Matrix effects and comparative study

Matrix effects were studied by spiking 100 ng/mL CYFRA 21-1 in six independent serum pools with initial CYFRA 21-1 concentrations ranging from 0.80 to 2.7 ng/mL. The calibration formula $y=\left(3.0x + 0.62\right) \cdot 10^{-2}$, obtained after linear least-squares regression, was used to calculate the measured values. The differences between the obtained values and the nominal concentrations were calculated and expressed as a percent deviation (% bias). The CYFRA 21-1 concentrations in serum of 60 randomly selected study participants (20 benign disease, 20 stage I-II NSCLC and 20 stage III-IV NSCLC) were measured using both IA LC-MS/MS and ECLIA and compared using a Bland-Altman plot to determine the analytical bias and limit of agreement (LoA) between the methods.

Receiver operating characteristics (ROC) curves were constructed to determine the area under the curve (AUC).

Results

Signature peptide selection

The tryptic CYFRA 21-1 peptides were evaluated based on their uniqueness in the human genome, peptide length, presence of the unstable amino acids and detectability after IA QTOF LC-MS$^5$ evaluation (Figure 1, Supplementary Figure 1). Peptide AALEDTLAETAR (amino acids 318–330) met all criteria and was selected as SP and AALEDTLAETEA\[R_{13C6, 15N4}\] was designed as IS. Optimal multiple reaction monitoring (MRM) transitions for both peptides were found to be 695.3>676.2 (y6 fragment, quantifier) and 695.3>1,005.4 (y9 fragment, qualifier) for the SP and 700.3>686.2 for the IS (Supplementary Table 1). The qualifier:quantifier ratio was used for quality control.

Evaluation of interference effects

During IA purification, an interference effect can be caused by non-specifically bound substances that hinder the binding of the target analyte to the antibody [27]. To elucidate the presence of potential interference effects, 100 ng/mL CYFRA 21-1 was spiked into four different matrices; PBS with 5% bovine serum albumin (BSA), BOV containing endogenous bovine CYFRA 21-1 which lacks the human SP sequence, purchased male AB serum (HUM-S) and four independent patient serum pools (HUM-P). IA LC-MS/MS analysis of these samples revealed large differences between the CYFRA 21-1 signals, expressed as the peak area ratio of the SP and IS, in the different matrices (Figure 2A). The maximum signal was obtained when using the least complex matrix (BSA), while the signal decreased by almost half upon the use of the purchased sera (BOV and HUM-S) and additionally halved in case of pooled patient serum (HUM-P). To confirm that the isolation efficiency decreased upon increased matrix complexity, the initial CYFRA 21-1 concentration spiked in the different matrices and the remaining CYFRA 21-1 concentration in the supernatant after IA purification was measured using ECLIA and used to calculate the percentage of uncaptured CYFRA 21-1. As shown in Figure 2B, a larger mean percentage of uncaptured CYFRA 21-1 remained in the supernatant of commercial serum (22 and 24 % for BOV and HUM-S respectively) compared to BSA (14 %). After isolation in patient serum, the highest percentage of CYFRA 21-1 remained in the supernatant (38 %). These results indicate the presence of significant interference effects during IA purification upon increased matrix complexity. No interference effects were seen during ECLIA (<20 %), which is expected to be a result of the low serum volume and large dilution factor used in this assay.

Common interferents are high abundant proteins including albumin and IgGs, which represent 60–90 % of the total serum protein concentration [28, 29]. As shown in Figure 2C, the albumin, IgG and total protein concentrations in HUM-S were indeed substantially lower compared to HUM-P (BOV could not be measured as the assays were compatible with human serum solely).

As the CYFRA 21-1 signal in BSA was substantially higher, albumin was not expected to cause the large interference effect in the serum matrices. To study the role of IgG, a monoclonal IgG1 antibody (cetuximab) dissolved in PBS was titrated in 100 ng/mL CYFRA 21-1, resulting in a large decrease in CYFRA 21-1 signal upon the presence of IgG (Figure 2D). This shows that IgG is significantly influencing the capture efficiency, which partially explains the differences between the studied matrices. The remaining interference effects, i.e. the difference between HUM-S and HUM-P, could not be assigned to specific proteins.

Protein precipitation

Multiple strategies were evaluated to decrease interference effects, such as ultrafiltration, IgG denaturation by disulfide reduction and several precipitation methods (data not shown). Only AS precipitation, which is a commonly used approach to salt out proteins [30], was compatible with the IA LC-MS/MS assay and successfully reduced the interference.
Here, HUM-S was spiked with 100 ng/mL CYFRA 21-1, precipitated using increased v/v percentages of AS, the pellet was dissolved in PBS and the CYFRA 21-1, albumin, IgG and the total protein concentrations were measured using commercially available assays. Optimal CYFRA 21-1 precipitation was achieved when using 50 % v/v AS, at which 9.1 % of the total albumin fraction, nearly all IgG and around 33 % of the total protein fraction was precipitated, which is in agreement with previous findings of high abundant protein precipitation (Figure 3A–D) [30, 31]. Since the IgG depletion pattern was similar to the one of CYFRA 21-1, AS precipitation could not solve all interference effects, e.g. caused by IgG. However, incorporation of AS precipitation in the IA LC-MS/MS assay significantly improved the absolute CYFRA 21-1 signal in HUM-P towards the same level as BOV (Figure 3E). This allowed the use of BOV as a surrogate matrix for calibration purposes, which is beneficial compared to human serum as it does not contain endogenous human CYFRA 21-1.

**Assay calibration and validation**

A calibration curve was analyzed on each of four subsequent days using weighted linear least-squares regression (1/x²). Linearity was achieved within the range of 1.0–100 ng/mL CYFRA 21-1 ($R^2=0.98$) (Figure 4A). The LLOQ was established at 1.0 ng/mL (Figure 4B). No carry-over was observed and blank BOV samples did not show a detectable peak at the retention time of the SP nor the IS thus ensuring high selectivity.
The within run and between run precision and overall accuracy were determined in 3 days, for which at each day 4 QC levels were analyzed in fivefold (Table 1). All precision and accuracy values were <15 %, which is well within the acceptance criteria of the EMA guidelines (<15 % for all QC levels except samples at the LLoQ, which should not exceed 20 %). At low QC level, the calculated between run mean squares was smaller than the within run mean squares, resulting in a between run precision of 0 %.

Matrix effects

LC-MS is known to suffer from matrix effects during ionization as a result of co-elution of other substances that can cause signal suppression or enhancement [32–34]. To this end, serum of six healthy individuals was spiked with 100 ng/mL CYFRA 21-1 and the calibration curve was used to determine the measured values. As shown in Table 2, all measured values were within tolerance criteria (<20 %). Of note, the detected values were slightly lower than the nominal concentration.

Comparative study

Samples of 20 patients with benign lung diseases, 20 stage I-II NSCLC patients and 20 stage III-IV NSCLC patients were analyzed with the developed CYFRA 21-1 LC-MS/MS assay and compared to a commercially available ECLIA. By combining
the analytical results, with exclusion of samples below the LLoQ of the LC-MS/MS assay (n=11), the analytical bias between the methods was determined to be $-3.3\%$ (LoA $-75$ and $69\%$), as shown in the Bland-Altman plot (Figure 4C). CYFRA 21-1, cytokeratin 19 fragment 21-1; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLoQ, lower limit of quantification; BOV, bovine serum; ECLIA, electrochemiluminescence assays; IA, immunoaffinity; LoA, limit of agreement.

Table 1: Precision and accuracy of the QC samples at LLoQ, low, medium and high concentration levels measured with five replicates at three different days.

<table>
<thead>
<tr>
<th>QC, ng/mL</th>
<th>Precision, % (CV)</th>
<th>Accuracy, % (bias)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLoQ (1.0)</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Low (3.0)</td>
<td>11</td>
<td>0.0</td>
</tr>
<tr>
<td>Medium (40)</td>
<td>3.6</td>
<td>7.5</td>
</tr>
<tr>
<td>High (80)</td>
<td>4.3</td>
<td>11</td>
</tr>
</tbody>
</table>

QC, quality control; LLoQ, lower limit of quantification; CV, coefficient of variation.

Figure 4: Analytical validation. (A) Calibration curve of CYFRA 21-1 (4 days). (B) LC-MS/MS quantifier signal at LLoQ (green, 1.0 ng/mL) overlaid with four blank BOV samples (gray). (C) Bland-Altman plot and (D) boxplots of the CYFRA 21-1 concentration in benign (n=20), stage I/II NSCLC (n=20) and stage III–IV NSCLC (n=20) patients measured using ECLIA and LC-MS/MS. %Difference$=100(\text{ECLIA} - \text{LC-MS/MS})/\text{average}$. CYFRA 21-1, cytokeratin 19 fragment 21-1; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLoQ, lower limit of quantification; BOV, bovine serum; ECLIA, electrochemiluminescence assays; IA, immunoaffinity; LoA, limit of agreement.
Table 2: Matrix effect tested by spiking six serum samples with 100 ng/mL CYFRA 21-1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measured, ng/mL</th>
<th>Bias, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99</td>
<td>-0.63</td>
</tr>
<tr>
<td>2</td>
<td>84</td>
<td>-16</td>
</tr>
<tr>
<td>3</td>
<td>89</td>
<td>-11</td>
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<td>4</td>
<td>90</td>
<td>-10</td>
</tr>
<tr>
<td>5</td>
<td>86</td>
<td>-14</td>
</tr>
<tr>
<td>6</td>
<td>96</td>
<td>-4.3</td>
</tr>
</tbody>
</table>

CYFRA 21-1, cytokeratin 19 fragment 21-1.

LC-MS/MS assay do not allow to draw significant conclusions on the diagnostic value of the assay.

Discussion

For the first time, a robust serum CYFRA 21-1 quantification method based on AS precipitation and IA LC-MS/MS analysis has been described. The power of combining AS precipitation and IA purification prior to LC-MS/MS quantification was demonstrated as it enabled removal of interfering components to minimize matrix- and interference effects and to lower the detection threshold. Good analytical sensitivity, accuracy and precision were obtained over the whole linear range in concordance with the latest EMA guidelines, therewith allowing for robust CYFRA 21-1 quantification.

A comparative study between LC-MS/MS and ECLIA showed good correlation between both assays, although some individual patient samples deviated >50%. This could be caused by the fundamental differences between both assays, including the required secondary antibody interaction in ECLIA and the different readout systems. Moreover, this study showed the presence of significant interference effects during IA purification that could partially be solved by AS precipitation. As ECLIA is also based on IA purification, the influence of interfering components should be considered, although they were not observed in this study. To further improve the reliability of the IA LC-MS/MS assay, a full-length protein IS would be beneficial. Unfortunately, a full-length protein IS is often not (commercially) available, as is the case for CYFRA 21-1. A peptide IS was therefore chosen as a generally accepted alternative strategy [34], as it enables correction of experimental variation during LC-MS/MS analysis, but cannot correct for experimental variation during sample workup.

The LC-MS/MS assay is expected to be more robust and reliable compared to ECLIA due to the improved detection specificity that MRM is offering. The diagnostic performance of the developed LC-MS/MS assay and ECLIA in discriminating NSCLC from benign lung diseases was similar, although no significant conclusions could be drawn due to the limited number of patient samples. Nevertheless, after further validation the LC-MS/MS assay could be the starting point for the development of a reference method for standardization of immunoassays, which is one of the most ongoing efforts in current clinical laboratories [35].

The optimized LC-MS/MS assay conditions and the analytical performance were similar to a previously published IA LC-MS/MS assay for the quantification of serum NSE [20], indicating that this assay format could serve as a template for the quantification of other protein TMs. The extra protein precipitation step prior to IA LC-MS/MS has not often been described, but was shown to improve sensitivity and reproducibility significantly. This therefore offers an advanced strategy for the LC-MS/MS-based quantification of low abundant serum proteins.

In conclusion, this novel AS precipitation, IA LC-MS/MS assay for the quantification of serum CYFRA 21-1 demonstrates the importance of proper sample workup to minimize matrix- and interference effects. The assay may aid in the exploration of the clinically added value of LC-MS/MS-based detection of serum CYFRA 21-1 compared to conventional immunoassays.

**Research ethics:** Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the Medical Research Ethics Committees United (NL58985.100.16). **Informed consent:** Informed consent was obtained from all individuals included in this study. **Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission. **Competing interests:** The authors state no conflict of interest. **Research funding:** The study was supported by The Netherlands Organization for Scientific Research (NWO) via LIFT grant 731.017.405. Roche Diagnostics partially sponsored the CYFRA 21-1 reagents used in the study. The funding organizations did not play a role in the design of the study, choice of enrolled patients, review and interpretation of data or preparation of approval of manuscripts. **Data availability:** Not applicable.

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