

CYFRA 21-I in urine: a diagnostic marker for endometriosis?

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Abstract: Diagnostic workup of endometriosis usually involves laparoscopic inspection and histological examination of biopsies. Unequivocal laboratory parameters for this ailment have not been available in routine diagnostic evaluations thus far. In this study, we examined urine concentrations of cytokeratin 19 (CYFRA 21-1), a structural protein specific for epithelia. We performed immunoassays for CYFRA 21-1 in urine samples from women afflicted with endometriosis throughout their menstrual cycle. We observed a significant increase in CYFRA 21-1 concentrations, corrected by creatinine levels, in the late follicular phase as compared with the level in healthy controls. We conclude that cyclically increased CYFRA 21-1 concentrations in urine could serve as a valuable noninvasive diagnostic parameter in the workup of clinically manifesting endometriosis.

Keywords: biomarkers, menstrual cycle, CYFRA 21-1, immunoassay, noninvasive diagnosis

Introduction

Endometriosis is one of the most common gynecological diseases, affecting 4%–15% of women of childbearing age.¹ Recognition and conclusive diagnosis only occur at a weighted average of approximately 9 years following the start of the disease, which undoubtedly contributes to the fact that 30%–40% of women suffering from endometriosis are considered infertile.^{2,3} Besides its clinical relevance, the disease has extensive psychological and economic ramifications.⁴ In addition, it is associated with an increased risk of developing various types of cancer.⁵

Of the women suffering from endometriosis, 60%–70% present with characteristic symptoms such as dysmenorrhea, dyspareunia, dyschezia, dysuria, menorrhagia, metrorrhagia, and cyclic back pain, as well as cyclic and noncyclic lower abdominal pain.⁶

Factors responsible for a delayed diagnosis include the following: asymptomatic course of disease, nonspecific symptoms, unremarkable findings in noninvasive examinations, and/or symptoms masked by contraceptive agents. Often, sociocultural factors play a role as well: women tend to trivialize menstrual pain because they often face lack of understanding within their families (familial clustering).^{7–9} The gold standard for a conclusive diagnosis is invasive laparoscopy with biopsy.

Noninvasive approaches to diagnostic workup have so far yielded merely marginally satisfactory results.^{10–17} These involve examinations of the following laboratory parameters.

Serum/blood

Cancer antigen 125 (CA 125), cancer antigen 19-9 (CA 19-9), interleukins, white blood cell (WBC) subpopulations are evaluated, and mRNA coding for chemokine receptor

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1 (CCR1) is carried out. Moreover, chronically elevated inflammatory parameters are found in endometriosis.

Urine

Analysis of urine peptide profiles using mass spectrometry is undertaken. Cytokeratin 19 (CK 19 or CYFRA 21-1) fragments are detected in urine in endometriosis, regardless of the stage of the menstrual cycle and creatinine levels.^{16,18}

CKs are cytoskeletal structures in eukaryotic epithelial cells. More than 20 CK subtypes are known. The filaments have a diameter of 6–25 nm and molecular weights ranging from 40 kDa to 67 kDa.¹⁹ CK1–CK8 belong to the neutral–basic type A subfamily, and CK9–CK20 belong to the acidic type B subfamily. In the filaments, the CK polypeptides are always paired so that a particular type A CK forms a complex with a particular type B CK, creating heterodimers.²⁰

CK 19 is present in virtually all epithelia. It is found in simple and stratified squamous epithelia as well as in, for instance, basal cells of mucous membranes.²¹ CYFRA 21-1 has a molecular weight of 40 kDa. It is composed of 400 amino acids and is found in high concentrations in the stratum functionalis of the endometrium as well as in menstrual blood.^{22,23} It is also present in the peritoneum and in the peritoneal fluid in lower concentrations. Ectopic endometrial tissue displays a high immunoreactivity for CYFRA 21-1.²⁴

It has been accepted that the expression of CYFRA 21-1 in suprabasal cells can be regarded as a marker of a precancerous process.²⁵ Various squamous cell cancers, such as lung cancer, intrahepatic cholangiocarcinoma, and urinary bladder cancer, are characterized by the release of CYFRA 21-1.²⁶ Apparently, as a result of tumor necrosis and the accompanying disintegration of cytoskeletal elements, elevated CYFRA 21-1 fragment levels are detected in serum.^{27,28} CYFRA 21-1 was detected in the urine and serum of patients with endometriosis as well. However, these observations were highly inconsistent, which is the reason why this marker has not been considered thus far.

The goal of our research was to investigate more precisely the CYFRA 21-1 levels in the urine of patients with endometriosis using a highly sensitive immunoassay. An issue of particular interest to us was menstrual cycle-dependent change.

Materials and methods

This study was proposed according to §15 of Bavaria's Code of Medical Ethics and was approved by the Ethics Committee of the Bavaria State Medical Association (file number 12074).

A total of 83 urine samples from 37 patients who presented at Munich's Center for Reproductive Medicine were analyzed (principal investigator: Dr U Noss). CYFRA 21-1 measurements were carried out in the Department of Laboratory Medicine of Wasserburg am Bodensee Medical Center (Bodenseelabor, Backenreuter Str 51, A-6912 Hörbranz, Austria). The average age of the patients was 36.2 years (standard deviation: 6.7, median: 36.0, range: 18–49), while the average age of the control group was 38.7 years (standard deviation: 4.0, median: 38.0, range: 32–45). At the time of urine sample collection, no patient was receiving hormone therapy. In accordance with the doctors' orders, this therapy was initiated only after the urine sample had been collected. The patients undergoing *in vitro* fertilization indicated their cycle day (CD), which was subject to a "close monitoring", including, *inter alia*, the cycle-dependent hormone levels. In the period from February 2013 to August 2013, midstream clean-catch urine samples were collected and stored at –25°C until analysis. In order to maintain the cold chain, the samples were shipped to the laboratory in Sarstedt® dry ice cold boxes. The samples were thawed at room temperature and gently shaken immediately prior to analysis. Maintaining the cold chain for CYFRA 21-1 is an absolute requirement.

The urine samples of endometriosis patients were divided into two groups: the follicular phase (FP) (n=17) samples and the luteal phase (LP) samples (n=19). The corresponding control groups comprised the following: FP samples (n=7) and LP samples (n=8). For 12 endometriosis patients and five control patients, urine samples from both phases of the menstrual cycle were available. Some urine samples consisted of an untreated sample plus a sample to which a proteinase inhibitor cocktail from Sigma-Aldrich company (catalog number P2714) was added as a sample matrix stabilizer at a concentration of 2 µL/mL prior to freezing.

CYFRA 21-1 immunoassay

CYFRA 21-1 levels were determined using a commercially available chemiluminescent microparticle immunoassay (CMIA) on the fully automated ARCHITECT i1000SR analyzer (Abbott, Wiesbaden, Germany) for the quantitative determination of soluble human CK 19 fragments. This assay is based on two monoclonal antibodies (mAbs) specific for CK 19: BM 19.21 and KS 19.1. This is a two-step immunoassay. In Step 1, the sample is mixed with paramagnetic microparticles coated with the KS 19.1 anti-CYFRA 21-1 mAbs. Next, a magnet draws the immune complex formed, and the unbound material is removed by means of a wash step. In Step 2, the labeled conjugate consisting of BM 19.21

anti-CYFRA 21-1 and the chemiluminescent acridinium ester is added. The labeled conjugate binds to the immune complex. Following another wash cycle to remove the unbound particles, the pretrigger (hydrogen peroxide) and trigger solutions (sodium hydroxide) are added to the reaction mixture. The chemiluminescence reaction takes place, yielding *N*-methylacridone, which releases energy in the form of light emission during the return to its original state (extraordinary analytical sensitivity!). The CMLA technology measures the emission over a predetermined time period in relative light units. This allows for the quantitative determination of analyte concentrations. The quantity of CYFRA 21-1 antigen in the sample is directly proportional to the relative light units.

The ARCHITECT CYFRA 21-1 assay is linear across the entire measuring range of 0.50–100.00 ng/mL. The sensitivity in terms of the limit of detection is 0.09 ng/mL, and the limit of quantitation is 0.17 ng/mL. This test is currently approved for the determination of serum CYFRA 21-1 levels in the diagnostic workup of cancer.

Creatinine determination

Creatinine in urine was measured using the Cobas c 111 System® (Roche Diagnostics, Mannheim, Germany) according to the Jaffé method by adding picric acid in an alkaline solution. CYFRA 21-1 levels were corrected using the creatinine value as the reference value of the sample matrix dilution ratio.

Statistical analysis

Statistical analysis was performed using the open source statistical software GNU R (version 3.0.2, 25 September 2013, www.r-project.de). The first step involved testing a random sample for normal distribution using the Shapiro–Wilk and Kolmogorov–Smirnov tests. Due to the absence of normal distribution, the comparison of CYFRA 21-1 values, paying due regard to menstrual cycle phases, was carried out using the Mann–Whitney *U*-test. The Wilcoxon test was run to test for a possible significant change in CK levels in patients in whom urine samples were collected on two occasions. If relevant, the negative and positive predictive values (NPV/PPV) were calculated using an estimated prevalence of 10%, with the aid of various random samples (Bayes' theorem) and the two-occasion urine sample combination and its percentage deviation. A *P*-value of 0.05 was considered statistically significant.

Results

This study involved examining urine concentrations of CYFRA 21-1 during the menstrual cycle of patients with

endometriosis. The measuring accuracy of the immunoassay system used was sufficient to be able to avoid repeat determinations. In endometriosis patients, a significant elevation in the urinary CYFRA 21-1 levels was observed, in particular, at the end of the FP as compared with healthy controls. In the LP, however, the CYFRA 21-1 levels dropped again. To avoid strong circadian fluctuations in CYFRA 21-1 levels, we had to use creatinine value as a reference value and correction factor.

We analyzed 17 random FP urine samples from 16 endometriosis patients. Due to the low number of cases, the area under a receiver operating curve was omitted and a cutoff value of >4 ng/mL/gCREA was adopted; 16 samples yielded pathological results (sensitivity =94.12%). The false negative (= nonpathological) sample was analyzed at an early point in the FP (CD 4), and the levels dropped by 48% in the LP (Figure 1).

We analyzed 21 random LP samples from 13 patients with endometriosis. Two samples could not be used due to the presence of bacteriuria (WBC +++ (enzymatic degradation of CKs). Using an adopted cutoff value of >4 ng/mL, six of the 19 samples yielded pathological results (sensitivity =31.5%). In four pathological samples, the urine was collected 1–2 days after ovulation. Another sample displayed a CYFRA 21-1 level of 4.6 ng/mL as far as into the 22nd day of the menstrual cycle (Figure 2).

In ten patients without endometriosis (ie, the control group), eight random urine samples from the FP and eight random urine samples from the LP were analyzed. Using an adopted cutoff value of <4 ng/mL, all samples yielded unremarkable results.

Sample collection on two different occasions was characterized by a highly significant difference (ie, a 61.31% drop) between the FP and LP titers in endometriosis patients. The value pair comprising CYFRA 21-1 levels of 9.34 ng/mL in the FP (CD 6) and 25.57 ng/mL in the LP (CD 18) was the only pair showing a titer increase (Figure 3). The change in the titer between the FP and LP was less pronounced in the control group (ie, 30.70%), including minimal changes in the case of low values (Figure 4).

Our preliminary tests have revealed that CYFRA 21-1 is very susceptible to degradation, even when using a proteinase inhibitor of the chosen concentration. Prompt freezing or immediate processing of the assay yielded the most optimal results. The testing procedure revealed that samples that were kept unrefrigerated for an extended period of time displayed severely reduced antigen contents, which could not be satisfactorily stabilized even with a proteinase

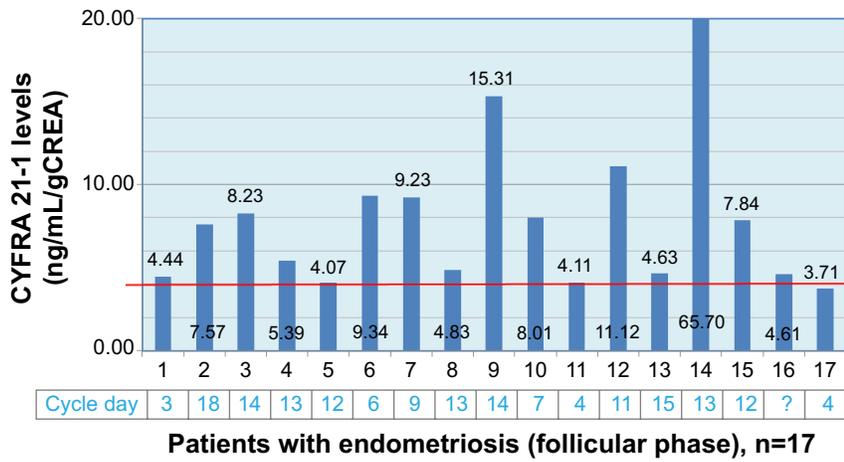


Figure 1 CYFRA 21-1 levels in patients with endometriosis (follicular phase).
Abbreviations: CYFRA 21-1, cytokeratin 19; CREA, creatinine.

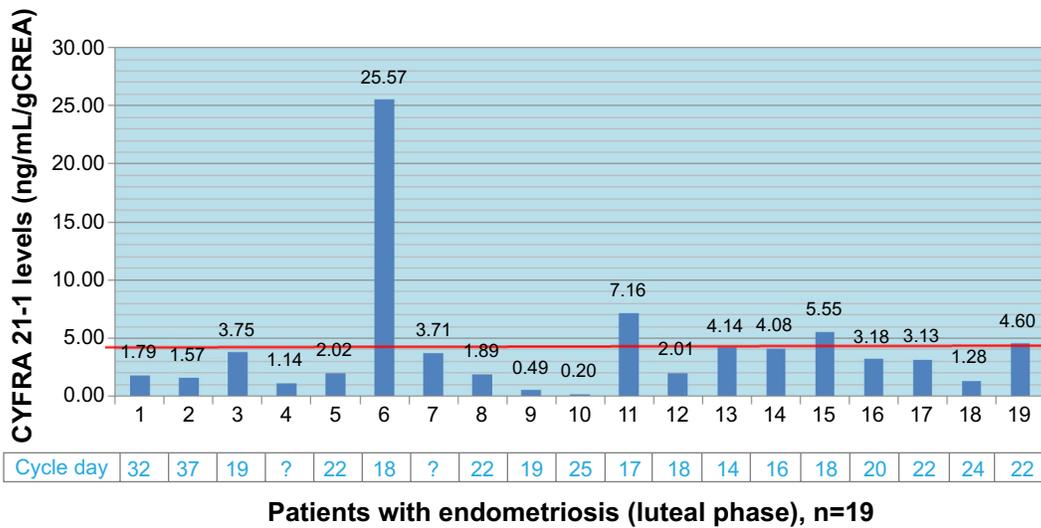


Figure 2 CYFRA 21-1 levels in patients with endometriosis (luteal phase).
Abbreviations: CYFRA 21-1, cytokeratin 19; CREA, creatinine.

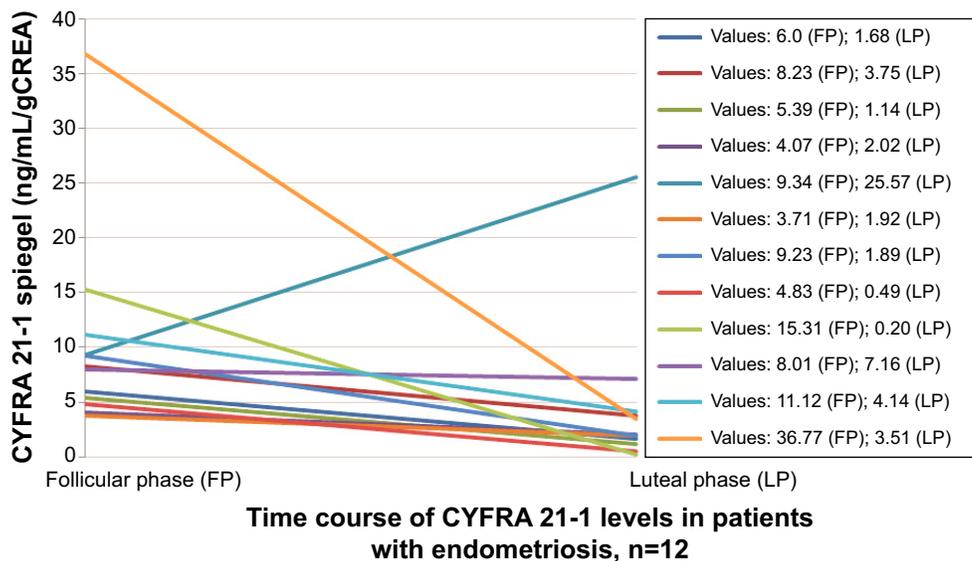


Figure 3 Time course of CYFRA 21-1 levels in patients with endometriosis.
Abbreviations: CYFRA 21-1, cytokeratin 19; CREA, creatinine.

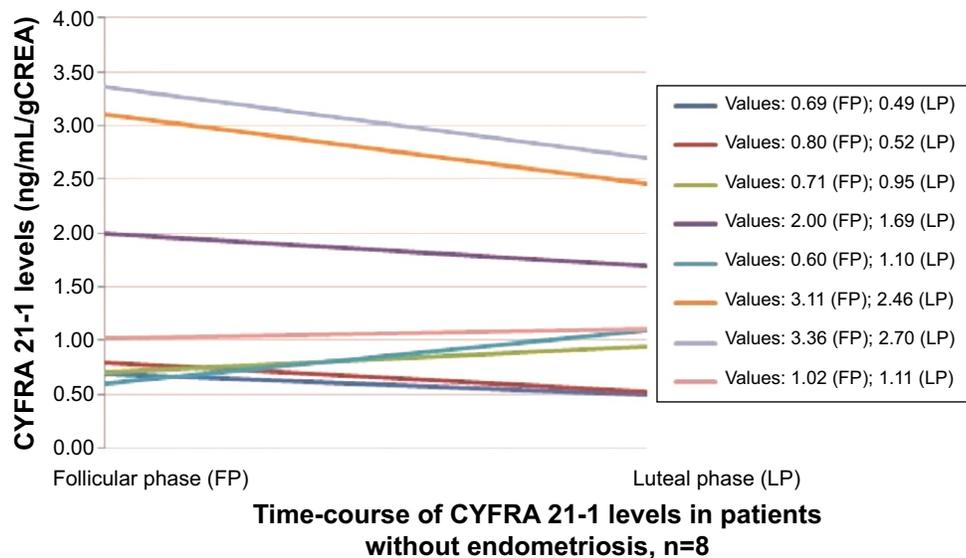


Figure 4 Time course of CYFRA 21-1 levels in patients without endometriosis. **Abbreviations:** CYFRA 21-1, cytokeratin 19; CREA, creatinine.

inhibitor. Centrifugation carried out prior to analysis led to a drop in CYFRA 21-1 levels of approximately 90%. Cells such as bacteria, WBCs, red blood cells, and/or epithelial cells contained in the urine accelerated the degradation of CYFRA 21-1.

Discussion

The highly significant increase in CYFRA 21-1 protein levels in the urine of women with endometriosis compared to the levels in women without this disease was also reported by Tokushige et al¹⁶ who performed Western blot analyses. However, neither our research group nor El-Kasti et al¹⁸ obtained confirmatory results in analyses of peptide profiles in urine that would support a cycle-dependent fluctuation in CYFRA 21-1 levels, which was assumed by Tokushige et al.¹⁶

Downregulation and upregulation of the expression levels of various proteins in the endometrium of patients with endometriosis have already been described in this context by several authors.^{29,30} Various noninvasive test methods using peripheral venous blood have been compared.¹² However, due to lack of sensitivity and specificity, they cannot as yet be recommended for routine use.³¹

Our study focused on the difference in the amount of CYFRA 21-1 excreted in the FP vs the LP. We were able to show the utility of performing measurements on different CDs in the diagnostic workup/ruling out of endometriosis. This approach took into consideration the cycle-dependent effects, particularly those of estrogen and progesterone, on endometriosis activity.

El-Kasti et al¹⁸ compared the renal peptide excretion profiles of the periovulatory phase with those of the LP, a highly relevant pursuit. Our observations in a patient who displayed a high 1-day postovulatory CYFRA 21-1 concentration (Figure 2, case 6) are in agreement with this. Another example is a patient from our study who displayed an early-FP (CD 4) CYFRA 21-1 level that was only slightly below our cutoff estimate of >4 ng/mL (Figure 1, case 17) and that altered the sensitivity from 100% to 94.1%. The percentage change in CYFRA 21-1 levels in urine samples taken on two different occasions during the menstrual cycle allows for the assessment of hormone sensitivity and increases the sensitivity and specificity beyond the cutoff value. This novel approach serves as a robust internal plausibility check for the measured CYFRA 21-1 levels and should accompany every interpretation of results in order to be able to factor in, inter alia, the nonspecific hormone-insensitive confounding factors (eg, bacteriuria, inflammations, etc).

To date, there has been no standardized protocol of urine sample preparation for CYFRA 21-1 measurement to achieve reproducible results. The first efforts in this direction were reported by Dittadi et al.³² Various preanalytical modifications were necessary to achieve reproducible and accurate results. The study design of Tokushige et al¹⁶ could not be used due to the highly significant drop (>90%) in CYFRA 21-1 levels following centrifugation of urine during sample preparation. When urine is centrifuged, CYFRA 21-1 with a molecular weight of 40 kDa accumulates in the sediment; hence, falsely low concentrations are obtained in the supernatant. In agreement with our observations,

Nisman et al³³ have pointed out that noncentrifuged urine samples lead to the best test results. Creatinine obligatorily had to be used as a correction factor. Circadian fluctuations in CYFRA 21-1 levels generally require creatinine as a corrective reference value. Creatinine correction may be dispensed with only in the case of 24-hour urine collection. Likewise, in a recently published study by Kuessel et al³⁴ in 2014, the cycle-dependent determination of CYFRA 21-1 levels in urine failed to bring a significant benefit for the diagnostic workup of endometriosis. This can be explained by the fact that an obligatory creatinine correction of catheter urine was not carried out. A valid analysis of the results of these studies is not possible due to these methodological errors. Finally, we refer to a recent publication by Lessey et al³⁵ who measured the CYFRA 21-1 levels in relation to creatinine. The cycle phases were not used, which is probably due to the fact that the patient samples dated back to the year 2011 and the cycle phases were not yet considered at that time.

Much is still unknown about the pathophysiological significance of CYFRA 21-1 in endometriosis.^{23,24} It still remains to be elucidated why it is sometimes detected in the urine when the corresponding serum levels are unremarkable.^{16,33} A possible explanation for this observation is that CYFRA 21-1 in serum acts as a substrate for enzymes such as, for instance, neutrophil elastase, and is therefore quickly degraded in inflammatory disease states such as endometriosis. Following excretion by glomerular filtration – CK 19 with a 40 kDa molecular weight – hardly any further enzymatic degradation takes place, thus making urine a better sample matrix.

Summary

The methodological errors clearly show that interdisciplinary teamwork is indispensable not only in daily practice but also in every analytical phase of clinical studies.

Further studies are needed to facilitate implementation of a noninvasive, effective, and cycle-dependent urine test for this enigmatic disease.

The new scientific findings call for cost–benefit analyses of screening methods for this highly prevalent disease, which could easily be diagnosed in its early, highly treatable stage.

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Disclosure

The author reports no conflicts of interest in this work.

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