

## Nucleosomes in Serum as a Marker for Cell Death

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The concentration of nucleosomes is elevated in blood of patients with diseases which are associated with enhanced cell death. In order to detect these circulating nucleosomes, we used the Cell Death Detection-ELISA<sup>plus</sup> (CDDE) from Roche Diagnostics (Mannheim, Germany) (details at <http://biochem.roche.com>). For its application in liquid materials we performed various modifications: we introduced a standard curve with nucleosome-rich material, which enabled direct quantification and improved comparability of the values within (CV<sub>intraassay</sub>:3.0–4.1%) and between several runs (CV<sub>interassay</sub>:8.6–13.5%), and tested the analytical specificity of the ELISA.

Because of the fast elimination of nucleosomes from circulation and their limited stability, we compared plasma and serum matrix and investigated in detail the pre-analytical handling of serum samples which can considerably influence the test results. Careless venipuncture producing hemolysis, delayed centrifugation and bacterial contamination of the blood samples led to false-positive results; delayed stabilization with EDTA and insufficient storage conditions resulted in false-negative values. At temperatures of –20 °C, serum samples which were treated with 10 mM EDTA were stable for at least 6 months. In order to avoid possible interfering factors, we recommend a schedule for the pre-analytical handling of the samples.

As the first stage, the possible clinical application was investigated in the sera of 310 persons. Patients with solid tumors (n=220; mean=361 Arbitrary Units (AU)) had considerably higher values than healthy persons (n=50; mean=30 AU; p=0.0001) and patients with inflammatory diseases (n=40; mean=296 AU; p=0.096). Within the group of patients with tumors, those in advanced stages (UICC 4) showed significantly higher values than those in early stages (UICC 1–3) (p=0.0004).

**Key words:** Cell death; Apoptosis; Nucleosomes; Histones; DNA; Serum.

**Abbreviations:** ABTS, 2,2'-azino-di-3-ethylbenzthiazol-

ine-sulfonate, AU, Arbitrary Units; CV, coefficient of variation; CDDE, Cell Death Detection-ELISA<sup>plus</sup>; *E.coli*, *Escherichia coli*; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; mU, absorbance units x 10<sup>-3</sup>; *S.aureus*, *staphylococcus aureus*; *P.aeruginosa*, *Pseudomonas aeruginosa*.

### Introduction

Nucleosomes are basic elements of chromatin. They are formed by a core particle which consists of an octamer of the histone components H2A, H2B, H3 and H4 and 146 base pairs of DNA which are wrapped around them. Fifteen to 100 base pairs of linker-DNA connect neighbouring nucleosomes and form a chain-like structure which is stabilized by a further histone H1 (1–3). During cell death, particularly during apoptosis, endonucleases are activated which bind specifically to the easily accessible linking sites and cleave the chromatin into multiples of oligo- and mononucleosomes. These are detectable in the cytoplasm at the early stages of the apoptotic cell death process (4–8). The degradation of the chromatin which occurs during oncotic cell death is less specific, but can also produce nucleosome-like fragments (4, 5, 9).

Nucleosomes which are released into circulation under physiologic conditions are quickly and effectively removed by hepatic metabolism or immunologic elimination and can only be detected in small amounts in the circulating blood (10–13). However, in the blood of patients with diseases which are associated with increased rates of cell death, such as the patients with malignant tumors, acute inflammation or autoimmune diseases, nucleosomes appear in elevated concentrations (14–22). This might reflect the high rate of spontaneous, as well as of therapy-induced, cell death (14–16, 19–22). Additionally, increased active release of nucleosomes by lymphocytes might contribute to the enhanced amount of circulating nucleosomes (23).

In former studies, free DNA was quantified in serum and plasma (14–16, 24, 25). As it was reported that most of the circulating DNA exists as oligo- and mononucleosomes (26, 27), we developed an easy-to-handle method for the direct quantification of the nucleosomes in serum or plasma. We used the Cell Death Detection-ELISA<sup>plus</sup> (CDDE) from Roche Diagnostics which originally was created as a cellular test system. As the applicability to liquid materials was insufficient, we performed various modifications and adapted the CDDE for use in serum samples.

Because of the quick elimination of nucleosomes from circulation *in vivo* (13) and the presence of endonucleases in serum *in vivo* and *in vitro* (28), the stability of circulating nucleosomes is limited. Other groups also

reported the low reliability of the determination of nucleosomes (29). We identified the pre-analytical handling of the blood samples as an essential factor which can considerably influence the test results. Inadequate blood drawing, sample transport, centrifugation, stabilization and storage conditions can provoke additional release of nucleosomes from blood cells, or promote nucleosomal degradation by endonucleases.

We identified possible interfering factors and developed a standardized schedule for the pre-analytical handling of samples. Together with the improved properties of the test system itself, these pre-analytical considerations seem to be prerequisites for the reliable determination of circulating nucleosomes in serum.

### Materials, Methods and Patients

We purchased the Cell Death Detection-ELISA<sup>plus</sup> (CDDE) from Roche Diagnostics (Mannheim, Germany) (No. 1774 425). The method and the materials used are described in detail at the Roche website (<http://biochem.roche.com>).

The assay is based on a quantitative sandwich enzyme-immunoassay principle. Two monoclonal mouse antibodies directed against DNA (ss- and ds-DNA) and histones (H1, H2A, H2B, H3 and H4) catch specifically mono- and oligonucleosomes which originate from the nucleus of eukaryotic cells. As mitochondrial, prokaryotic, viral DNA and RNA are not associated with histones and do not show nucleosomal arrangement, they are not detected by the CDDE.

#### Materials

The materials for the CDDE were prepared as indicated at the Roche website (<http://biochem.roche.com>) and included the following:

- PBS (Phosphate Buffered Saline): Ready to use solution containing 8.0 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, x 2 H<sub>2</sub>O, 0.2 g KH<sub>2</sub>PO<sub>4</sub>.
- Incubation Buffer: Ready to use solution containing phosphate buffered saline with 1% BSA, 0.5% Tween 20 and 1 mM EDTA.
- Anti-Histone-Biotin: Tablet of lyophilized biotinylated anti-histone-antibodies (monoclonal antibodies from mouse clone H11-4) which was dissolved in 450 µl distilled water (50 µg/ml).
- Anti-DNA-POD: Tablet of lyophilized anti-DNA-antibodies (monoclonal antibodies from mouse clone M-CA-33) with activated peroxidase which was dissolved in 450 µl distilled water (5 units/ml).
- Immunoreagent: 1/20 volumes of anti-DNA-POD and 1/20 volumes of anti-histone-biotin were thoroughly mixed with 18/20 volumes of incubation buffer before use.
- Substrate Buffer: Ready to use solution.
- Substrate: 3 tablets of 2,2'-azino-di-3-ethylbenzthiazoline-sulfonate (ABTS) which were dissolved in 15 ml of substrate buffer immediately before use.
- Streptavidin-coated microtiter plate.

#### Assay procedure

Twenty µl of the sample (serum, diluted 1:4 with incubation buffer) were placed into the streptavidin-coated microtiter plate. Subsequently, 80 µl of the immunoreagent (see above) were added; the microtiter plate was covered with an adhesive foil and incubated for 2 hours on a microtiter plate shaker

(500 g). During the incubation period, the anti-histone antibody reacted with the histone-component of the nucleosomes. Simultaneously, it fixed the immunocomplex to the streptavidin-coated microtiter plate by its biotin-component. Additionally, the peroxidase-labeled anti-DNA antibody bound to the DNA component of the nucleosomes. After removal of unbound antibodies by a washing step (3 x 300 µl incubation buffer), the amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex: the fixed complexes were incubated with 100 µl ABTS on a plate shaker at 250 g. The substrate reacted with the peroxidase, resulting in color development proportional to the amount of nucleosomes captured in the antibody sandwich. After 30 min of color development, the absorbance was measured photometrically at 405 nm against substrate solution as a blank (reference wavelength 492 nm).

#### Matrix

We used serum as matrix because of its good stability and the planned automation of the test. Ten ml of blood were collected by venipuncture in caolin-affected tubes (Kabe) which are used in clinical routine for serum investigations. One to two hours after venipuncture, the samples were centrifuged at 3000 g for 10 min. Subsequently, 1/10 volumes of 100 mM EDTA (in TRIS buffer, pH adjusted to pH 8) were added to 9/10 volumes of serum and mixed thoroughly.

Two hundred µl of the treated sera were placed in 2 ml tubes and stored at -20 °C. After thawing, the samples were mixed by vortex for 3 s. Subsequently, they were diluted with incubation buffer (1/4 volumes of serum and 3/4 volumes of incubation buffer) and mixed once again by vortex for 3 s. Afterwards, 20 µl of the diluted samples were placed into the microtiter plate.

#### Patients

We investigated sera from 310 persons: 50 healthy individuals, 40 patients with acute inflammation and 220 patients with solid tumors, among them 34 with lung cancer, 55 with colorectal cancer, 41 with other gastrointestinal cancers, 51 with breast cancer and 39 with ovarian cancer. The concentration of nucleosomes in serum was quantified before the patients underwent the recommended therapy (in malignant tumors mostly surgery) to estimate the spontaneous rate of cell death; 206 of these 220 patients with solid tumors had complete staging investigations and were included in the evaluation according to tumor stages.

### Results

First investigations revealed deficiencies of the original version of the CDDE regarding the comparability between different microtiter plates which also were observed by other groups (29). Probably, the discrepancy was caused by 1) the lack of a standardized time of color development or, alternatively, a stop reagent; 2) the use of an enrichment factor for the interpretation of the results which based on a negative control with a high interassay variation; and 3) the lack of a standard curve.

#### Standardization of the color development

First, we fixed the time of color development at 30 min. Thus, the theoretical intraassay error which was due to different periods of ABTS incubation because of man-

ual performance of the assay, was decreased to less than 5%.

#### Standard curve

To enable reliable quantification of nucleosomes, we established a standard curve using a reference material which contained high amounts of nucleosomes. Ten ml of EDTA blood from three healthy persons were mixed and incubated at 37 °C (5% CO<sub>2</sub>) for 24 hours. After centrifugation, the nucleosome-rich supernatant was lyophilized and stored at 4 °C. After resuspension and 1:24 dilution with incubation buffer, after 30 min of ABTS incubation the material reached reliable values of about 2500 mU which is in the upper measuring range of the photometer. Further dilution (1:24, 1:32, 1:48, 1:64, 1:96 and incubation buffer only) resulted in a linear curve running through the origin. Nucleosomes were quantified in Arbitrary Units (AU): after 30 min of ABTS incubation, 1000 AU corresponded to 2500 mU,

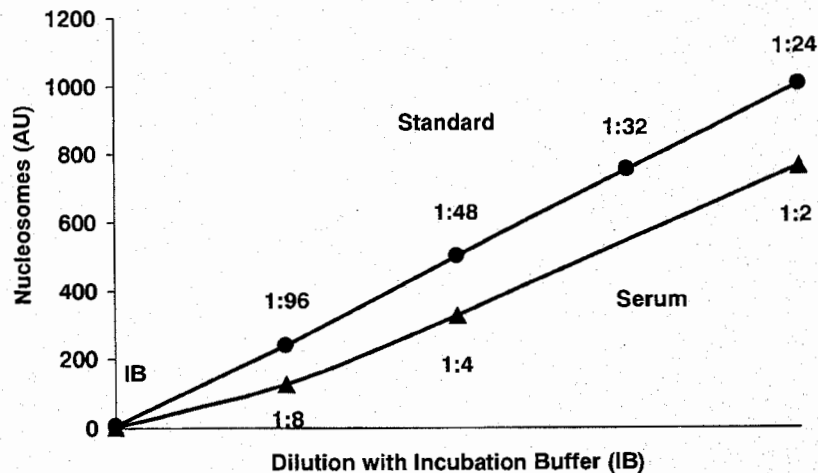
500 AU to 1250 mU, 200 AU to 500 mU, *etc.* Whereas the absorbance values measured in mU increased during the color development, the values in AU were stable because of their direct relationship to the standard curve.

Similarly to the standard curve, the dilution of serum with incubation buffer (1:2, 1:4, 1:8, 1:16, incubation buffer only) resulted in a linear and proportional curve. Because many of the native serum samples exceeded the measurable range at an ABTS incubation time of 30 min, we diluted all samples 1:4 with incubation buffer (see above; Figure 1).

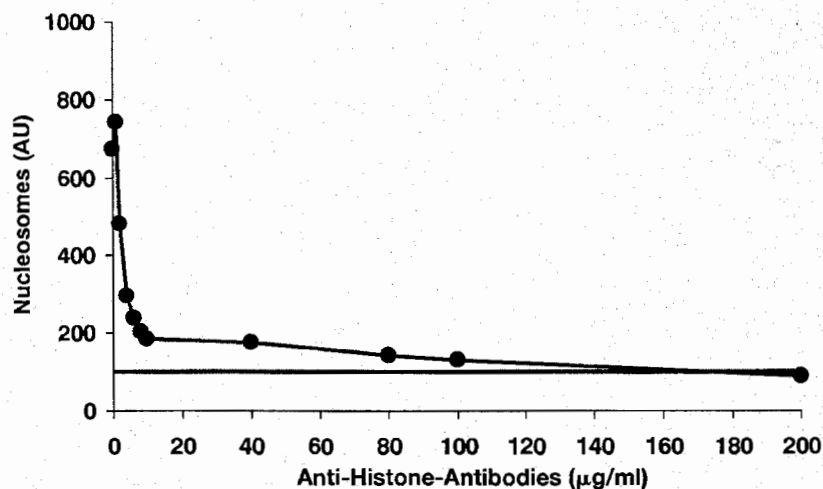
The lowest detection dose (LDD) was calculated at 38 mU=16 AU using the formula: mean value (n=20)+3 x standard deviation of the blank.

#### Analytical specificity

We titrated native anti-histone antibodies to the normal immunoreagent which contained biotinylated anti-his-



**Fig. 1** Standard curve for the detection of nucleosomes in serum. The standard curve as well as the serum dilution curve are linear and proportional lines which pass through the origin.



**Fig. 2** Analytical specificity. Anti-histone antibodies are added by titration to the standard immunoreagent containing biotinylated anti-histone antibodies. The measured signal which decreases in a concentration-dependent manner into

the range of healthy persons (<100 AU) reflects the exclusive quantification of complexes which are formed from biotinylated anti-histone antibodies, nucleosomes and peroxidase-labeled DNA antibodies (see text).

**Tab. 1** Intra- and interassay variation of nucleosome measurement.

	Intraassay n=10	Interassay n=14
Pool 1	$\bar{x}$ =327 AU CV=3.0	$\bar{x}$ =455 AU CV=8.6
Pool 2	$\bar{x}$ =181 AU CV=4.1	$\bar{x}$ =235 AU CV=13.5
Pool 3	$\bar{x}$ =47 AU CV=12.4	$\bar{x}$ =24 AU CV=46.0

tone antibodies and found declining absorbance values with increasing concentrations of the titrated native anti-histone antibodies. This observation indicated 1) that there was competition between native and biotinylated anti-histone antibodies, 2) the absence of other interfering factors within the test system, and 3) that only nucleosomes which are bound to peroxidase labeled anti-DNA antibodies and biotinylated anti-histone antibodies were detected (Figure 2). DNA, histones, nucleosomes, anti-histone antibodies or anti-DNA antibodies alone could not produce a detectable signal.

#### Imprecision

In order to estimate the reliability of the measurements within one run and between several runs, we investigated the imprecision using three pools with high (455 AU), intermediate (235 AU) and low (24 AU) absorbance values. At high and intermediate levels, the intraassay coefficient of variation (n=10) ranged between 3.0% and 4.1%, the interassay coefficient of variation (n=14) between 8.6% and 13.5%. In lower ranges of absorbance values, the coefficients increased to 12.4% in the intraassay and to 46.0% in the interassay comparison (Table 1).

#### Stability

Because of the limited stability of nucleosomes in serum or plasma, the choice of the matrix and the pre-analytical handling of the samples deserves particular emphasis: in the first step, we compared the serum and EDTA-plasma matrices of 10 persons (4 healthy persons, 4 patients with malignant tumors and 2 patients with acute infections) and investigated the influence of storage conditions such as storage time, temperature and addition of a stabilizer (10 mM EDTA to pH 8).

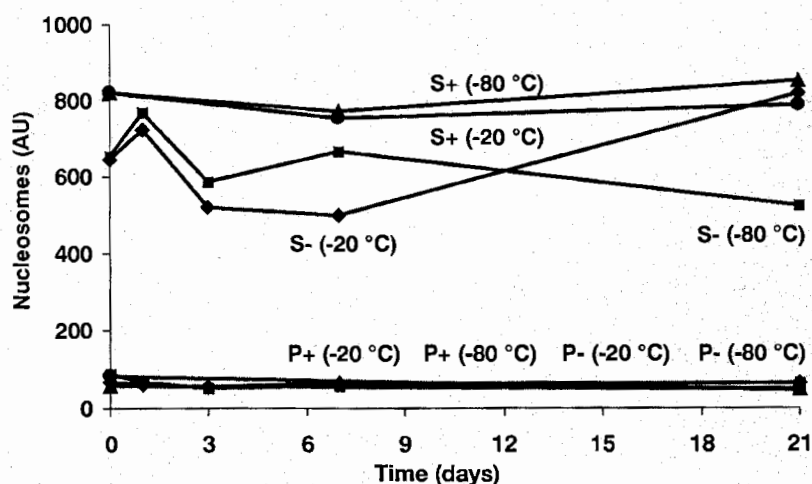
We found in most cases (n=9) higher absorbance values in serum than in plasma. Only in one patient with sepsis, values in both serum and plasma reached high levels.

These serum and plasma samples were separated into two groups with and without treatment with 10 mM EDTA (pH 8). All samples were stored for 3 weeks at 25 °C, 4 °C, -20 °C, and -80 °C. The best stability was observed for serum treated with 10 mM EDTA (pH 8) after centrifugation and stored at -20 °C or -80 °C (Figure 3).

Further, we investigated the pre-analytical handling in detail, looking for possible adverse factors which would affect the serum samples 1) between venipuncture and centrifugation, 2) between centrifugation and EDTA addition, 3) between EDTA addition and freezing, 4) during long-term storage and 5) between thawing and test performance.

1) After venipuncture, we stored the samples at 37 °C, 25 °C and 4 °C for 0, 2, 4 and 6 hours before centrifugation, treated them with EDTA immediately after centrifugation and subsequently stored them at -20 °C. With increasing delay before centrifugation, the absorbance values increased considerably. This effect was particularly strong at a storage temperature of 37 °C and less prominent at 25 °C and 4 °C.

2) After centrifugation (1 hour after venipuncture), the serum samples were stored at 37 °C, 25 °C and 4 °C before 10 mM EDTA (pH 8) was added after 0, 2, 4, 6 and 8 hours. Subsequently, they were frozen at -20 °C



**Fig. 3** Type for sample and sample storage conditions for nucleosome detection. Comparison between measurements in serum (S) and plasma (P), with (+) and without (-) 10 mM

EDTA (pH 8). Samples were stored at various temperatures (here shown for -20 °C and -80 °C).

(S1-S3). With increasing delay before adding EDTA, the absorbance values decreased in a time-dependent manner. The storage temperature had no impact on these values (Figure 4).

3) After centrifugation (1 hour after venipuncture) and subsequent treatment with 10 mM EDTA, we stored the serum samples at 37 °C, 25 °C and 4 °C for 0, 2, 4 and 8 hours before freezing them at -20 °C (E1-E3). Neither the storage period before freezing nor the temperature influenced the measured values (Figure 4).

4) We investigated the long-term stability using three serum pools with low (55 AU), intermediate (297 AU) and high (481 AU) absorbance values after storage periods of 1, 2, 3, 4 and 6 months at -20 °C. The values remained constant, which was indicated by coefficients of variation between 4.2% and 9.2% (Figure 5).

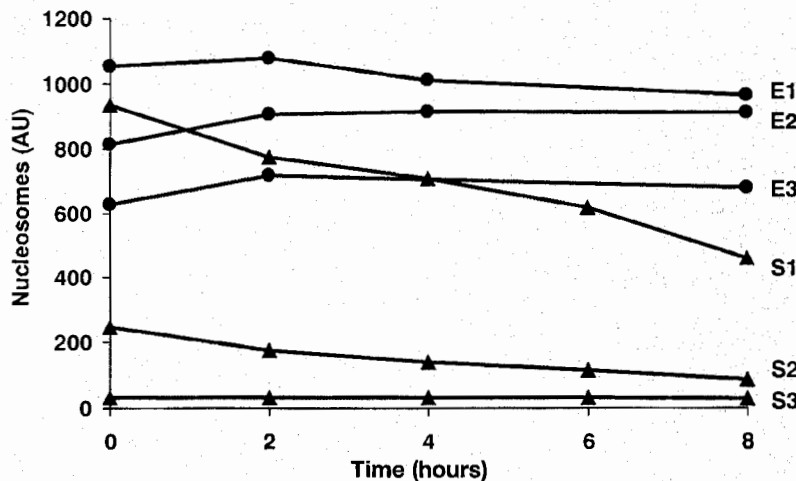
5) As 1:4 dilution of the serum with incubation buffer was required, we investigated whether the dilution before and after freezing produced comparable absorbance values. At various levels, samples which were

diluted before freezing showed values about 50% lower than samples which were diluted immediately before measurement (Figure 6). Moreover, we performed three freeze-thaw cycles with two serum samples and observed slightly increasing absorbance values. These results underlined the necessity to perform the dilution of the samples only immediately before measurement and to avoid the refreezing of the samples.

After thawing, the serum samples were centrifuged at 1600 *g* for 1 min or vortexed for 1, 3, 5, 10 and 20 s. There were no differences in the resulting absorbance values and the procedure was standardized to 3 s vortexing.

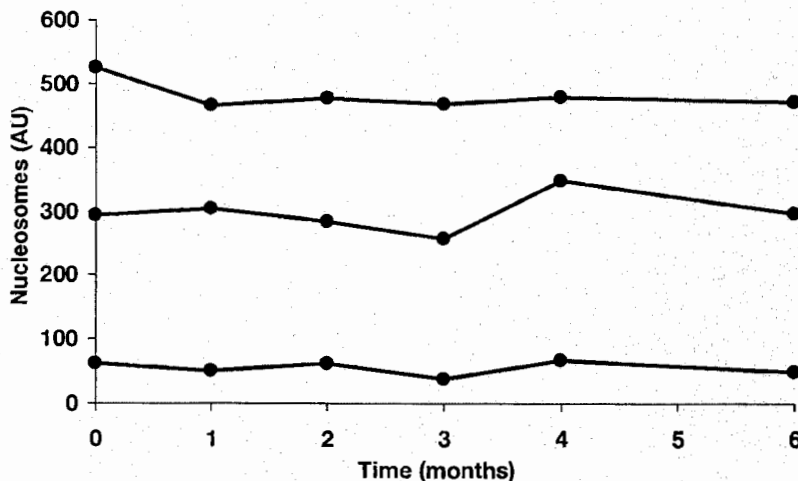
#### *In vitro influences*

We induced gentle hemolysis in a blood sample by shaking and by exposition to 37 °C for 4 hours. After centrifugation we spiked 250  $\mu$ l serum with absorbance



**Fig. 4** The effect of EDTA on nucleosome measurement. Sera which were stored at 4 °C after centrifugation and to which 10 mM EDTA (pH 8) was added (S1-3) after various periods, exhibit a decrease in the measured values depending

on the delay time. Sera to which 10 mM EDTA (pH 8) was added immediately after centrifugation and which subsequently were stored for various periods at 4 °C (E1-3) show stable values.



**Fig. 5** Long-term stability of samples. Sera with low, medium and high concentrations of nucleosomes exhibit a good stabil-

ity over at least 6 months after adding 10 mM EDTA (pH 8) and storage at -20 °C.

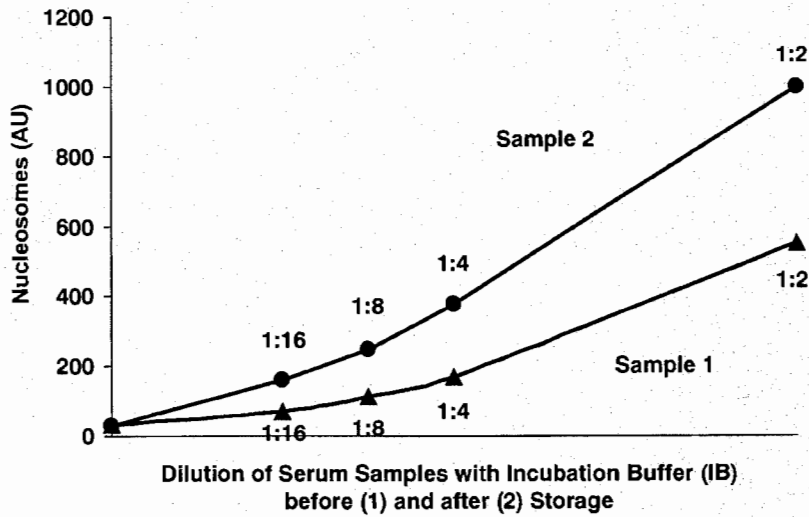
values below the lowest detection dose, with serum from the hemolyzed blood in increasing amounts (1  $\mu$ l, 2  $\mu$ l, 3  $\mu$ l, 5  $\mu$ l, 10  $\mu$ l). The values of the spiked serum increased proportionally to the concentration of the added hemolyzed serum.

In a further experiment, we tested whether only DNA of eukaryotic cells which is arranged in nucleosomes is detected by CDDE or whether also prokaryotic DNA of bacterial origin leads to measurable signals.

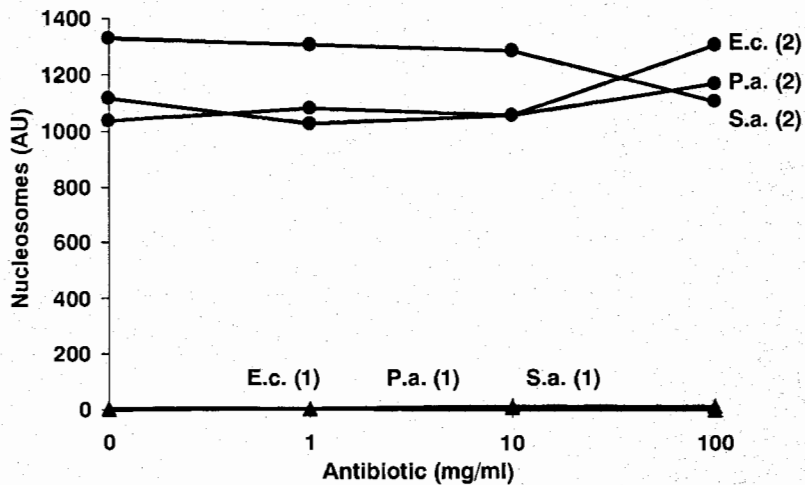
Several concentrations ( $10^2$  cells/ml,  $10^5$  cells/ml,  $10^8$  cells/ml) of *Escherichia coli* (*E.coli*), *Staphylococcus aureus* (*S.aureus*) and *Pseudomonas aeruginosa* (*Paeruginosa*) preserved in the Ringer solution were incubated for 4 hours with antibiotics in increasing doses (*E.coli* and *S.aureus*: Cefotaxim 1, 10 and 100 mg/ml; *Paeruginosa*: Ceftazidim 1, 10 and 100 mg/ml). Subsequently, the concentrations of nucleosomes

were determined in the supernatants. Even after killing the highest concentration of bacteria with the highest dose of antibiotics which should have resulted in high amounts of DNA in the supernatant, we did not observe any detection signal in the CDDE.

The same bacteria ( $10^2$  cells/ml,  $10^5$  cells/ml,  $10^8$  cells/ml of each *E.coli*, *S.aureus* and *P. aeruginosa*) were incubated in whole blood samples of a healthy donor for 4 hours with the same concentrations of antibiotics. Subsequently, the samples were centrifuged and the nucleosomes were determined in the sera of these samples. We observed very high values in the CDDE for all concentrations of bacteria and all concentrations of antibiotics. These results suggest that bacterial DNA itself cannot be detected by the CDDE, but, when incubated in human blood, it can lead to a massive stimulation of cell death or to the active release of



**Fig. 6** The effect of sample dilution before storage. Sera diluted with an incubation buffer mixture before storage (sample 1) show considerably lower measured values than sera immediately diluted before measurement (sample 2).



**Fig. 7** The effect of bacterial contamination on nucleosome measurement. Various concentrations (here shown for  $10^5$  bacteria/ml) of *E. coli* (*E.c.*), *S. aureus* (*S.a.*) and *P. aeruginosa* (*Pa.*) were incubated in the Ringer solution with various concentrations of antibiotics (*E.c.* and *S.a.*: 1, 10 and 100 mg/ml Cefotaxim, *Pa.*: 1, 10 and 100 mg/ml Ceftazidim) for 4 hours resulting in measured values at the lowest detectable dose (1). Incubating the same bacteria and antibiotics in human blood, the measured values reached high levels independently of the concentration of the added antibiotics (2) (see text).

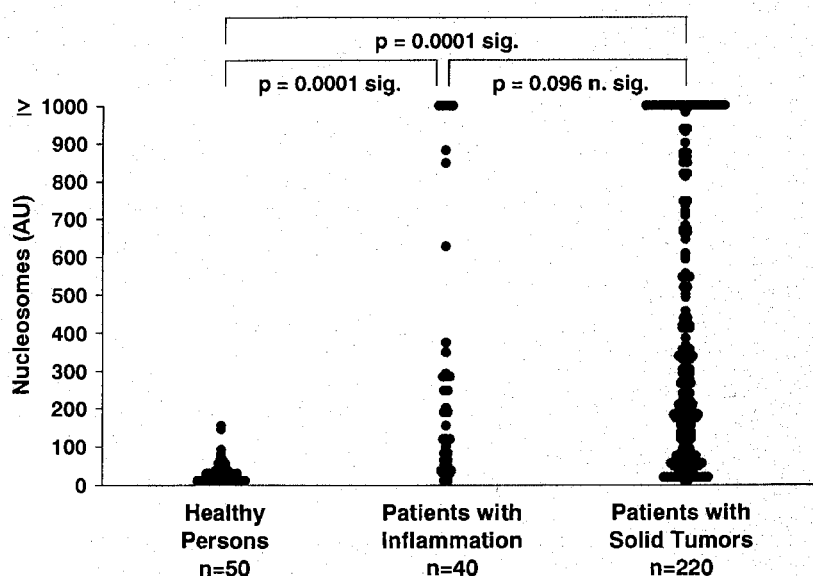
nucleosomes (Figure 7).

#### Clinical applicability

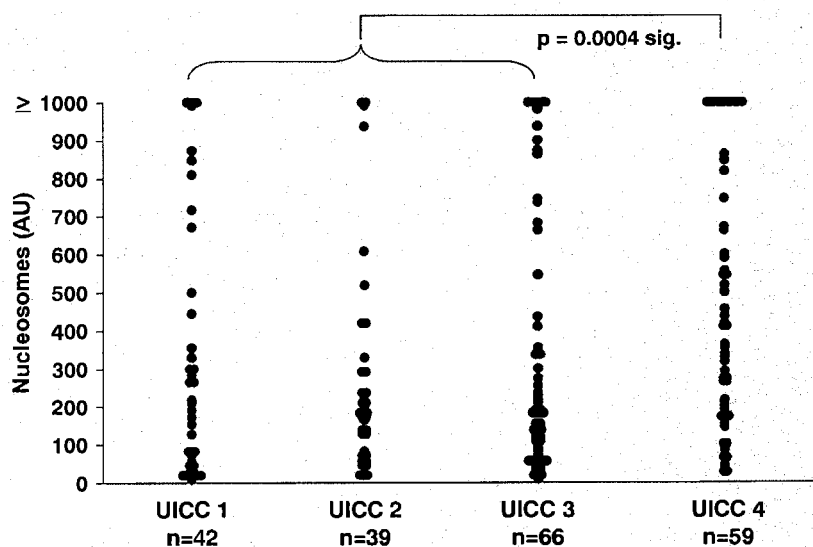
The concentration of nucleosomes was very low in serum of almost all healthy persons. It ranged from <16 AU to 150 AU with a median of 23 AU and a mean of 30 AU. In contrast, patients with solid tumors showed values ranging from low to very high (<16 AU to 1260 AU). The median of all tumor patients was 210 AU and the mean 361 AU. Among the various tumor entities, lung cancer was associated with the highest levels of nucleosomes in serum (median 357 AU, mean 524 AU), whereas the lowest levels were observed in colorectal cancer (median 151 AU, mean 310 AU). Also in patients

with acute inflammatory disease, we found a broad range of values (<16 AU to 1249 AU). The median reached 134 AU and the mean 296 AU (Figure 8). Whereas the discrimination between healthy persons and patients with solid tumors as well as between healthy persons and patients with inflammation was statistically significant ( $p=0.0001$  Wilcoxon test), it did not reach the level of significance observed between the patients with tumors and inflammatory disease ( $p=0.096$  Wilcoxon test).

Within the group of patients with solid tumors, advanced stages (UICC 4) were associated with significantly higher values than early stages (UICC 1-3;  $p=0.0004$  Wilcoxon test) (Figure 9). This phenomenon was most evident in patients with gastrointestinal tu-



**Fig. 8** Distribution of the spontaneous concentrations of nucleosomes in serum of healthy persons, patients with acute inflammatory disease and patients with various solid tumors.



**Fig. 9** Distribution of the spontaneous concentrations of nucleosomes in serum of patients with various solid tumors according to the tumor stage.

mors, whereas many patients with lung cancer and breast cancer had already high values at early stages.

## Discussion

The modifications of the Cell Death Detection-ELISA<sup>plus</sup> (CDDE), including the standardization of the ABTS incubation time at 30 min and the establishment of a standard curve with AU, considerably enhanced the intraassay (CV<10%) and interassay (CV<15%) precision of the CDDE and enabled a reliable and reproducible quantitative determination of the concentration of nucleosomes in liquid materials.

Nucleosomes in serum mostly occur in the form of mono- and oligonucleosomes in unknown proportions (30). Depending on their arrangement in mono- or oligonucleosomes (with or without tertiary structure), they might have different accessibility to antibodies (5). This means that the number of peroxidase-labeled anti-DNA-antibodies does not necessarily reflect the exact concentration of the nucleosomes. Therefore, we determined the nucleosomes with a relative scale in AU instead of absolute and possibly erroneous concentrations in ng/ml nucleosomes.

Nucleosomes were detected in several matrices: in serum, plasma, urine and cerebrospinal fluid (for urine and cerebrospinal fluid: authors' unpublished results). In agreement with other studies on free DNA (16, 24, 25), we found higher concentrations of nucleosomes in serum compared to plasma. This phenomenon might be explained by the stress exerted on blood cells during the clotting process before centrifugation which causes additional apoptosis and enhanced release of DNA and nucleosomes (24). Moreover, plasma samples contain chelators which inhibit the activity of the Ca<sup>2+</sup>- and Mg<sup>2+</sup>-dependent endonucleases. Subsequently, the cleavage of chromatin into oligo- and mononucleosomes, and the disclosure of antibody binding sites is impaired. Additionally, plasma proteins might react with nucleosomes and mask their presence in plasma. For our investigation, we used serum as a matrix, considering its good stability after treatment with 10 mM EDTA (pH 8).

The time-dependent increase in the nucleosome values which was due to the delay between venipuncture and centrifugation could be explained by the induction of apoptosis *in vitro* during clotting and the additional release of nucleosomes. Furthermore, endonucleases potentially start cleaving the tertiary structure of chromatin and uncover formerly hidden antibody binding sites, which lead to a stronger detection signal by the peroxidase-labeled anti-DNA-antibodies.

In contrast, the destruction of antibody binding sites at the ends of the nucleosomes by endonucleases which are still present in serum might be the reason for the time-dependent decrease in the absorbance values in case of delayed addition of EDTA after centrifugation (28). EDTA in a concentration of 10 mM seems to inhibit effectively the activity of the Ca<sup>2+</sup>- and Mg<sup>2+</sup>-dependent endonuclease, DNase I. pH 8 neutralizes the acidifica-

tion which often accompanies cell death and creates unfavorable conditions for DNase II, which is active at pH 4.5 (31). If EDTA is added to serum immediately after centrifugation, further fragmentation of nucleosomes by endonucleases seems to be sufficiently suppressed.

Exposition of the blood samples to heat or shaking were other stress conditions for the blood cells and often produced hemolysis. Additional cell death *in vitro* and release of nucleosomes count for the false-positive values in these cases.

High values were also obtained after incubation of bacteria and antibiotics in full blood samples of healthy persons. The signal could theoretically be caused by prokaryotic DNA which was released from lysed bacteria or by high amounts of nucleosomes from blood cells which were attacked by bacteria or damaged by antibiotics. As bacteria which were incubated in neutral medium and lysed by antibiotics did not cause an increase in the values, we could show that 1) prokaryotic DNA does not interfere with the ELISA and 2) the signal in the samples from healthy individuals incubated with bacteria and antibiotics was probably due to *in vitro* death of blood cells such as macrophages and polymorphonuclear neutrophils (32, 33).

In order to avoid those possibly interfering influences, we developed a standardized schedule for the pre-analytical handling of blood samples as follows:

1. Careful venipuncture without producing hemolysis.
2. Centrifugation within 1 to 2 hours after venipuncture.
3. Addition of 10 mM EDTA immediately after centrifugation.
4. Storage at -20 °C on the same day, and storage at -80 °C for periods longer than 6 months.
5. Homogenization by vortex for 3 s after thawing.
6. Dilution with incubation buffer immediately before performing the test.

The possible clinical relevance of the CDDE was shown by studying serum samples from persons with different diseases. Whereas these first results show healthy persons as a homogeneous group with very low values, patients with acute inflammation as well as those with malignant diseases, show a broad range of values. The concentration of nucleosomes in serum might reflect the rate of cell death (17, 34) and the acuteness of the process. In patients with malignant tumors, it probably corresponds with tumor type, activity and mass, and is related to the variable degree of perfusion and to the inter- and intratumoral heterogeneity of cells to undergo cell death (14-16). However, high cellular turnover at early stages can lead to high levels of nucleosomes in some tumor types. In acute inflammation, the role of the acute-phase proteins has to be also considered. They are able to bind to the anionic histone component of the nucleosomes and delay their elimination from the circulation (35, 36).

Other studies concerning the concentration of circulating nucleic acids in plasma and serum, although measured by different techniques, show comparable results for various tumors (14-16).



## Conclusion and Perspective

The modified version of the CDDE is a sensitive, reliable and reproducible method to quantify the concentration of nucleosomes in serum. The standardized pre-analytical handling of blood samples prevents the effect of interfering factors. The spontaneous concentration of nucleosomes might reflect the rate of cell death particularly in active, well perfused tumors and in acute inflammatory processes. Follow-up studies of such patients on and off therapy might be useful for estimating the extent and activity of solid tumors, as well as the sensitivity to therapy and the therapeutic efficacy.

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