MedInnovation
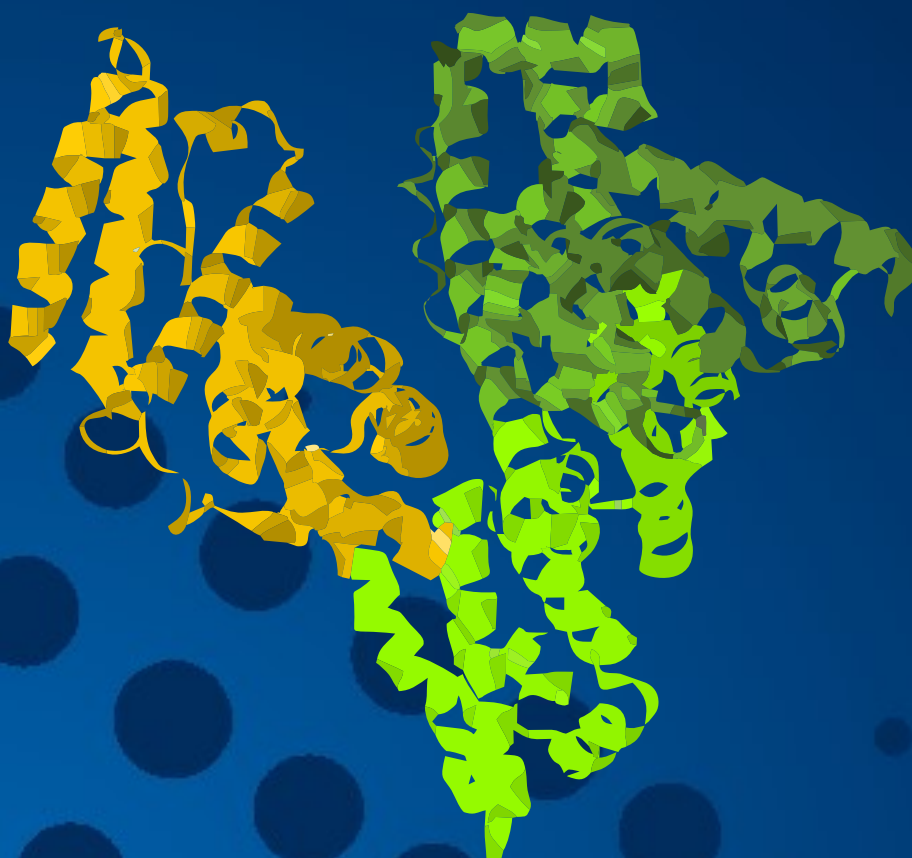## Advanced Albumin Research

Analysis of functional characteristics of serum albumin
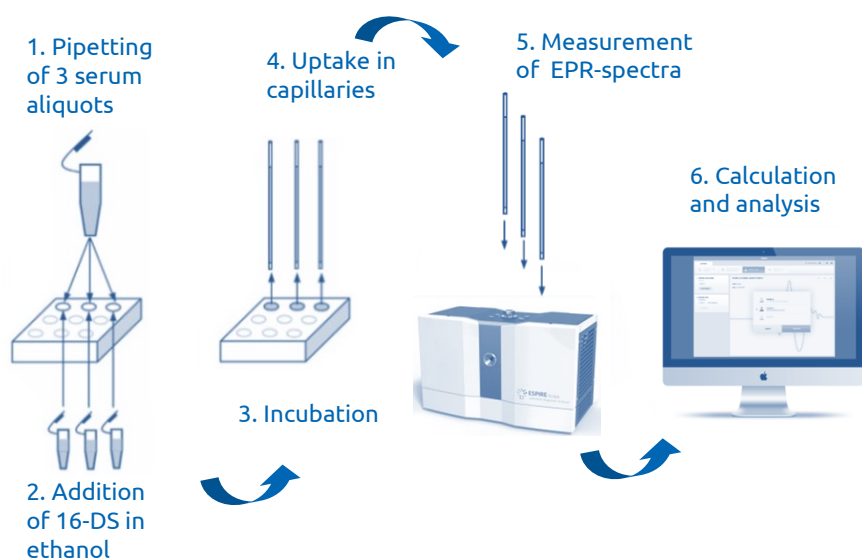
Principles - Albumin

Albumin is the most abundant protein in human blood serum. It is produced in the liver and has a serum half-life of approximately 19 days.

Transporting a large variety of hydrophobic substances like fatty acids, drugs and meta-bolites is one of its main physiological functions [1].

Beside this it maintains the oncotic pressure and buffers the pH of the blood.

For long chain fatty acids seven binding sites are known [2], three of them with high and four with lower affinity [3]. The binding sites with high affinity are described as long and narrow pockets, whereas those with low affinity are short and wide [2].

During the last years low molecular weight biomarkers bound to serum carrier proteins like albumin were intensively investigated, assuming they might have a potential for early disease detection [4, 5, 6].

1. Pipetting of 3 serum aliquots
2. Addition of 16-DS in ethanol
3. Incubation
4. Uptake in capillaries
5. Measurement of EPR-spectra
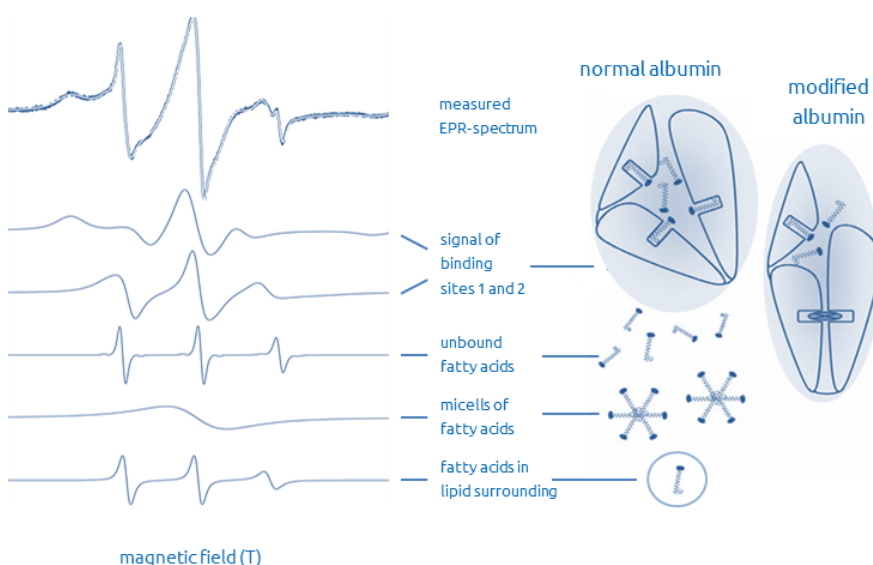6. Calculation and analysis

EPR technology

The albumin-functionality-test uses electron paramagnetic resonance spectroscopy (EPR) to estimate the functionality of albumin in human serum. This platform solution consists of different modules for different diseases.

It is based on a comparison of three different albumin-ethanol-fatty acid solutions, which simulate binding, transport and release conditions in vitro [7, 8]. By adding a spin-labelled fatty acid, the binding sites of albumin can be investigated.

Binding constants, binding capacities and biophysical parameters of both binding site types in the three different serum-ethanol-fatty acid solutions can be estimated by simulating the EPR spectra and hence the transport parameters (BE = binding efficiency, RTQ = real transport quality, DTE = detoxification efficiency) can be calculated.

measured EPR-spectrum
signal of binding sites 1 and 2
unbound fatty acids
micells of fatty acids
fatty acids in lipid surrounding
magnetic field (T)
normal albumin
modified albumin

Equipment

ESPIRE Scope
Laboratory Diagnostic Analyzer

- Applicable in standard laboratory routine – easy to handle.

- Automated device – generating parameter control algorithms, automated measurement procedure, signal registration and pre-processing of spectra as an integral process.

- Provides high accuracy, stability and sensitivity – at a high throughput rate.

- Guarantees comparable results in the analysis of several aliquots of one sample.

- Especially designed for the analysis of samples of biologic materials, where molecular conformation changes depending on temperature, pH and other factors occur.

- All algorithms programmable and provide a wide range of routine as well as scientific applications.

ESPIRE AlbuMini
Automated Liquid Handling System

- Accurate contact and non-contact sample preparation system for handling of chemical or biological solutions or suspensions

- Avoids human pipetting errors, shows highly reproducible results and increases productivity

- High resolution touch panel display with an intuitive user friendly interface

- High precision of pipetting with less than 2% (10-14µl)

Diagnostic Kit

- Set of solutions of 16-doxyl-stearic acid in ethanol (three different concentrations with different cap color)
- 96-well microtiter plates for sample incubation
- Lid for microtiter plate
- Glass capillaries
- Wax on undercoat for capillary sealing
- Laboratory film for microtiter plate's wells closure during incubation
- Package

Applications

- Cancer diagnosis & monitoring

- Quality control of commercial albumins

- Estimation of albumin transport and detoxifi-cation parameters in patients with several diseases

Criteria for exclusion

Acute exacerbations of chronic inflammatory diseases, such as Crohn's disease, ulcerative colitis or rheumatoid arthritis distort test results.

For regeneration of serum albumin it is necessary to keep a time lag of four weeks before blood sampling.

After surgery, chemo and/or radiation therapy, it is also necessary to keep a time lag of four weeks before blood sampling. This also applies to diseases with inflammatory processes.

Because at the moment the albumin-functionality-test does not give an information about the location of the cancer, it is inadvisable to use it in a screening situation.

For an adequate analysis we require:

- At least 2 ml blood stored at 8°C. The whole blood should attain to MedInnovation GmbH within 24 hours after sampling.

- "Pure" (centrifuged) serum should attain to us within at least 4 days after sampling. Centrifugation should be done at 1,000 – 1,500 x g at room temperature. Serum or EDTA plasma must be transferred into a separate and labeled tube.

- Blood withdrawal systems with anticoagulant (except EDTA) or gel are not allowed and if possible please don't use vacuum collection systems.

- Storage of samples for more than 4 days must be at less than -25°C, repeated freeze-thaw cycles of serum / plasma is not permissible.

- Study sample, which will be stored for more than 3 month should be stored at temperature less than -35°C, better at -80°C

- It is allowed to have a light breakfast.

- Blood withdrawal should be done in the morning.

Modified binding characteristic can be detected by Electron Paramagnetic Resonance Spectroscopy (EPR).

An integral discrimination function (DR – **D**iagnostic **R**esult) can be calculated from the parameters achieved from the EPR spectra.

In case of a DR value less than 1.0 the albumin conformation has changed and there is a very high probability of an active malignant process.

In case of a DR value higher than 1.0 a high probability of no malignant disease exists. In a threshold range from 0.8 to 1.2 is still a high risk of the presence of a malignant disease.

In clinical studies the albumin-functionality-test showed – in dependence of the location – a high sensitivity (about 90%) and specificity (about 90%) [10 - 14].
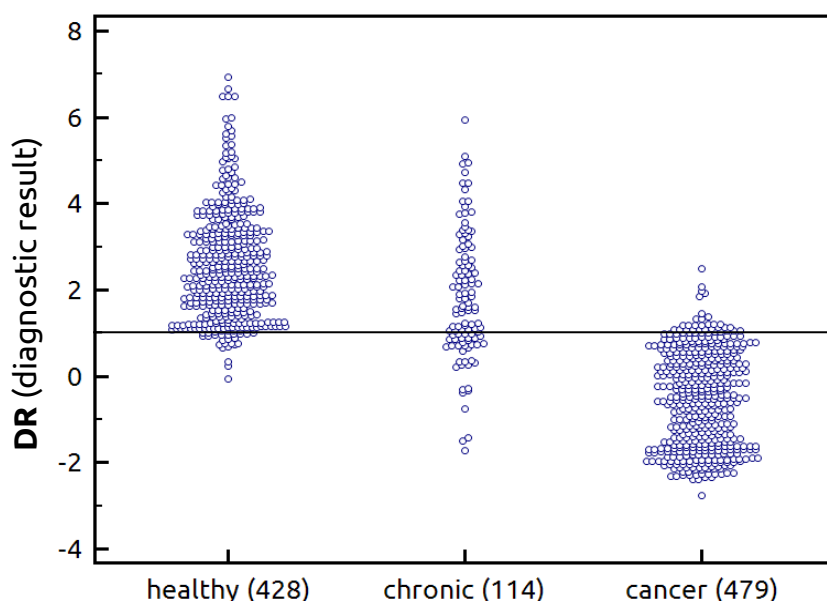
Fields of application
- therapy monitoring and relapse control
- early detection of cancer in populations with elevated risk (contact with carcinogenic substances, accumulation of cancer in family history)

Albumin-functionality-test

The albumin-functionality-test offers a possibility for easy and fast detection of active tumor growth, independently from type or location.

The variety of albumin function for all transport stages is based on the high conformational flexibility of this protein.

In the case of cancer, several specific peptides and lipids are present in the blood circuit in very low concentrations [5, 9].

Some of these molecules preferentially accumulate to serum albumin and influence the albumin function by changing its conformation.

Under pathological conditions there are several modifications of conformation mobility of albumin, resulting in modified transport and altered detoxification characteristics.

A comparison of the structure of normal albumin and albumin saddled with cancer peptides or lipids reveals a dramatic extent of conformational changes.

Comparison with tumor markers

In a study investigating patients with diseases of the large bowel the albumin-functionality-test was compared to the tumor marker CEA (Carcinoembryonic antigen) and CA 19-9 [13].

In case of colorectal carcinoma the albumin-functionality-test showed clear advantage in sensitivity (91%) compared to conventional tumor markers CEA (46%) and CA 19-9 (28%).
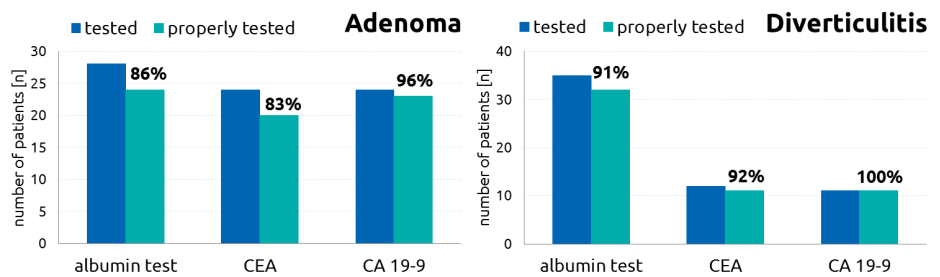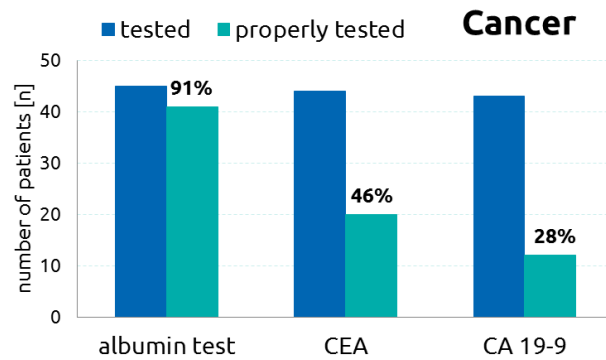

In case of adenoma and diverticulitis the albumin-functionality-test and the conventional tumor marker CEA and CA 19-9 are comparable in their specificity.

Patients with colorectal diseases

Studies with patients with malignant and benign colorectal diseases were conducted in Berlin, Bochum and Moscow.

A clear correlation between the DR value and the malignancy and the risk to malignant degeneration is recognizable.

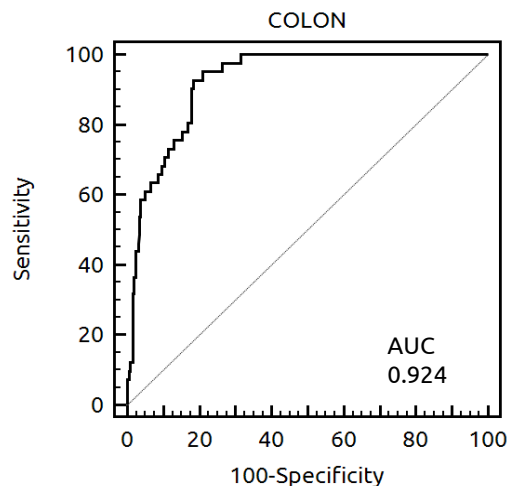Further investigations are on the way to enhance the number of cases to verify the first results.
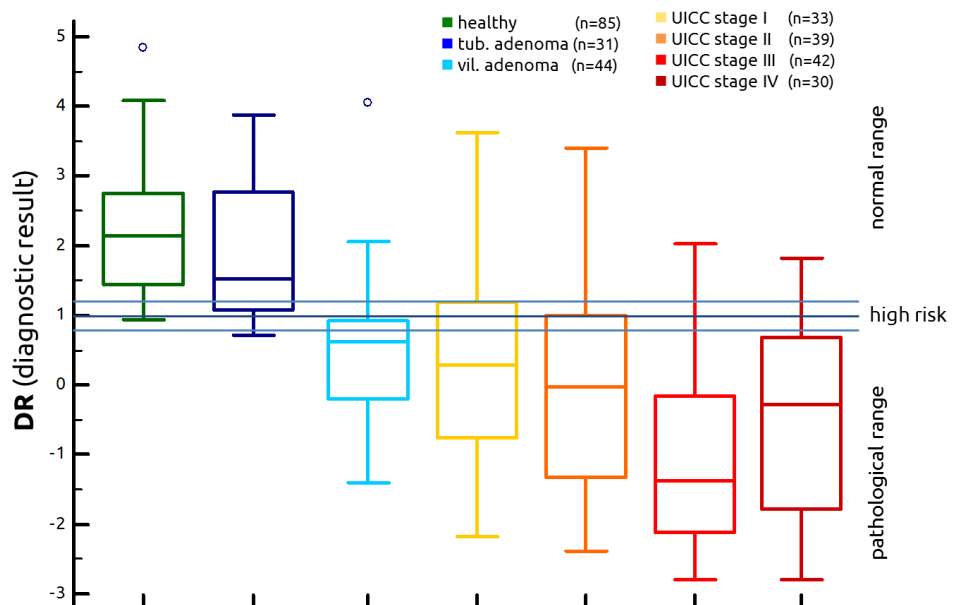
After a validation study regarding benign and malignant colorectal diseases, the albumin-functionality-test could be used for early detection of colorectal cancer in risk groups and for prolongation of colonoscopy intervals. [DRKS-ID of recent study – DRKS00025249]

First results for determination of cancer location

In a proof of concept project we have developed algorithms for four cancer types (colon, pancreas, prostate and mamma carcinoma).

For the classification of colon cancer the ROC curve is shown, consisting of 41 colon cancer cases and 322 patients with either pancreas, prostate or mamma carcinoma, as well as patients with benign colorectal diseases and healthy controls.

COLON

Sensitivity / 100-Specificity

AUC 0.924

First analyses of biophysical parameters achieved from EPR spectra have shown that a discrimination of different tumor localizations is possible.

Further investigations with larger groups and more cancer localizations are on the way to verify these first results.
[DRKS-ID of recent study – DRKS00025249]

Albumin-functionality-test improves clinical diagnosis of prostate cancer

A comparison of ROC analysis of free-to-total PSA ratio (%PSA) and a parameter combination of %PSA, total PSA, albumin concentration and DR (model) of 60 prostate cancer patients and 99 age-matched healthy controls is shown.

Sensitivity / 100-Specificity

- - - %PSA   AUC=0.746 SE=0.041
—— model AUC=0.901 SE=0.024
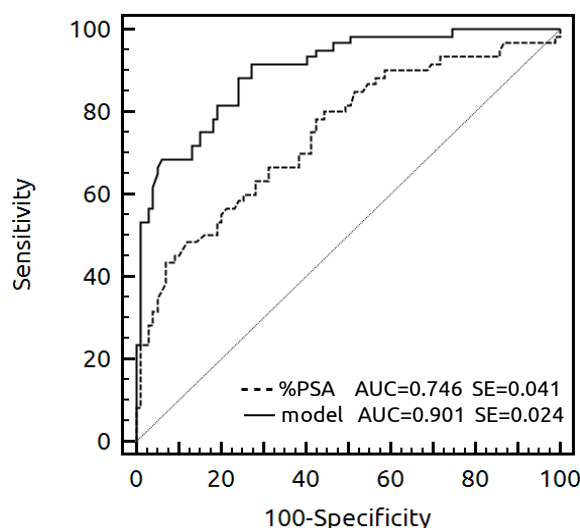
The Study was conducted with the Department of Oncology and Cancer Epidemiology University Hospital Lund, Sweden (unpublished data).

It is possible to increase the efficiency of prostate carcinoma diagnosis without invasive method by a parameter combination of PSA related values and the albumin-functionality-test.

All values could be ascertained from the same blood sample.

7

Rationale of recent study (DRKS-ID – DRKS00025249)

The results presented above are validated in a current, extensive study.

The group of cancer patients is included in order to determine the sensitivity and specificity of the Albumin-functionality-test as function of cancer localization and to optimize the algorithms for the localization.

The correlation of the DR value with the tumor stage or tumor size is analyzed and compared with established tumor markers.

The additional use of the transport parameters to optimize the diagnostic performance is analyzed.

The group of benign diseases and cancer-healthy probands makes it possible, among other things, to check the model for the detection of prostate cancer.

In this multicenter, prospective study (in the area of Berlin-Brandenburg) 1500 test persons are examined with the albumin-functionality-test. The study population is divided into three different groups.

The group of patients with benign diseases should reflect the clinical application situation in which medical professionals need to differentiate between benign and malignant.

The group of healthy people, with the same age and gender structure as the other two groups, is required to determine the specificity, which is also necessary in the situation of relapse control in asymptomatic patients.

Healthy group (probands without cancer)

- 300 healthy probands
- Without any inflammatory diseases in the past four weeks
- Without cancer or cancer therapy in the last two years
- Wit the same age and gender distribution as in the other two groups

Cancer group

- 500 patients with therapy naive cancer
- without any cancer therapy/invasive diagnostic or inflammatory diseases in the past four weeks
- with one of the followed cancer localization: breast, prostate, lung, colon, uterus, stomage, urinary bladder, kidney, pancreas or Non-Hodgkin-lymphoma (NHL)
- each cancer localization should include approximately 50 patients with the same distribution of staging

Group with benign diseases

- 700 patients with benign diseases of same organs as the cancer patients, reflecting the clinical situation in which the test is to be used (suspect for malignancy)
- At least 50 patients should be included for each organ
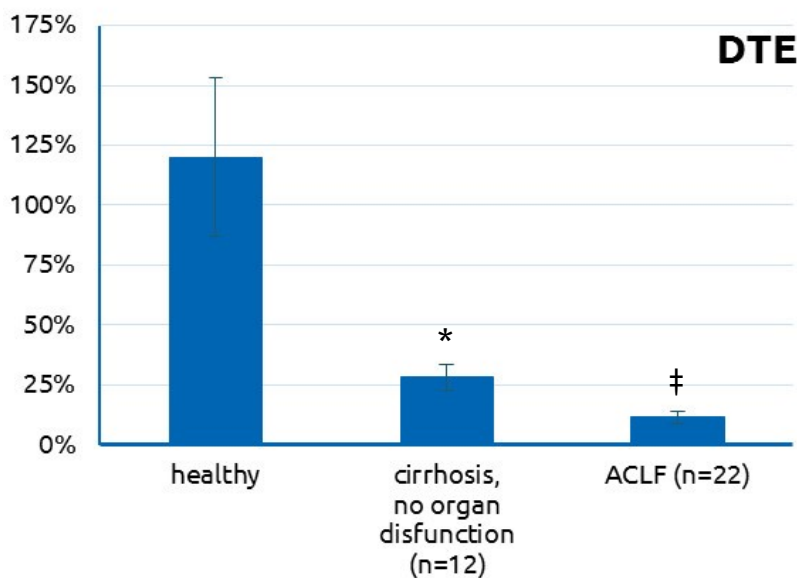
Liver diseases

Detoxification function

Binding of toxins and metabolites

Transport to liver

Detoxification in liver

Study results

In a study investigating patients with different liver diseases, the albumin-functionality-test showed that the detoxification efficiency of these patients is distinctly reduced, in comparison to a healthy control group [15, 16].

In patients with acute-on-chronic liver failure (ACLF), this parameter showed a further reduction in comparison to patients with liver cirrhosis without organ dysfunction.

* p<0.001 compared with healthy volunteers
‡ p<0,01 compared with cirrhosis

Studies on liver diseases are currently being carried within the context of clinical studies [study #1 University of Bologna, Sant'Orsola Malpighi University Hospital Ethics Committee code 075/2012/U/Tess; study #2 ANSWER-Study (EudraCT, number 2008–000625–19, and ClinicalTrials.gov, number NCT0128879) ; study #3   UCL Medical School London, Projekt ALIVER: DIALIVE -ACLF NCT03065699].

Transport parameters

The albumin-functionality-test based on EPR technology provides different parameters for comprehensive evaluation of the albumin functionality.

The **detoxification efficiency** (DTE in %) is a functional parameter for evaluation of the quality of albumin as transport vehicle in competitive situations.

It describes how well toxins can be eliminated and harmful substances can be delivered to the target tissue, also if there exists an increased accumulation of this substances in the organism.

Additionally the **binding efficiency** (BE in %) describes capacity of the fatty acid binding sites and the **real transport quality** (RTQ in %), the transport function of the investigated albumin solution.
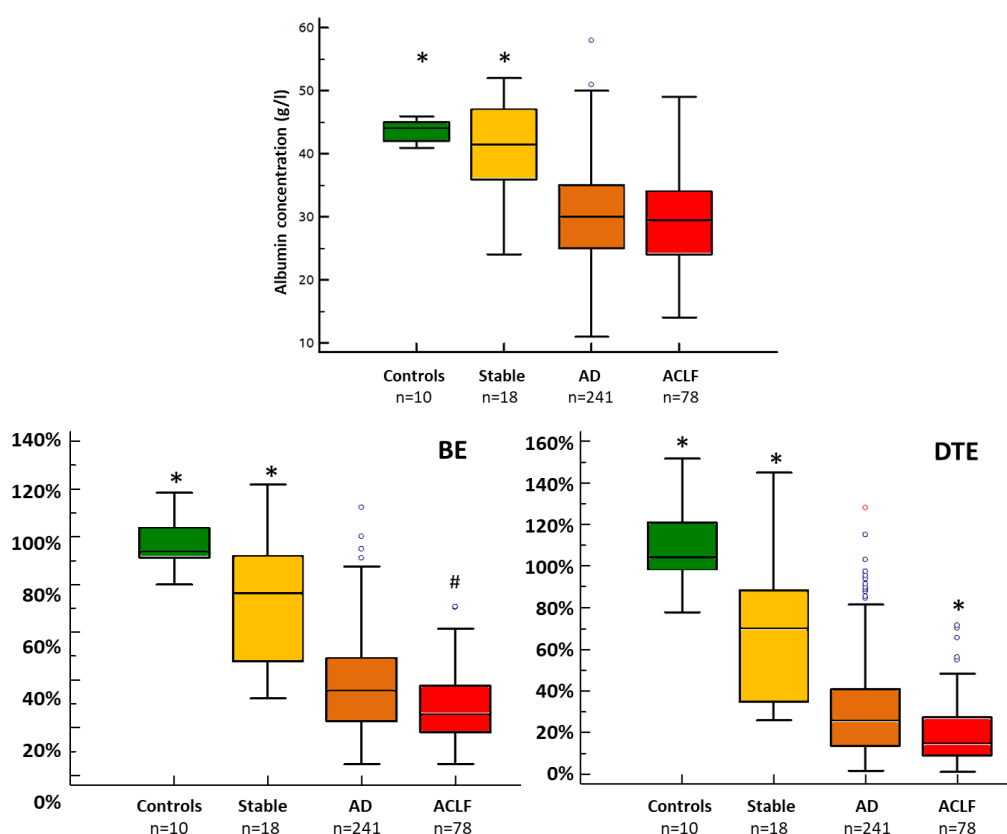
Publication Hepatology 2021

The latest data from the albumin function test in patients with liver disease were published in Hepatology in 2021 together with clinicians and scientists from the University of Bologna, Italy („Determination of Effective Albumin in Patients With Decompensated Cirrhosis: Clinical and Prognostic Implications.")

In this study, non-hospitalized patients with stable cirrhosis (stable) were compared with hospitalized patients with acut decompensated cirrhosis (AD) or ACLF with regard to their albumin (concentration and quality).

The albumin-functionality-test was able to show in patients with acut decompensated cirrhosis (AD) or ACLF that the binding and detoxification efficiency is significantly reduced compared to the control group but also to patients with stable cirrhosis (stable). It is also possible to differentiate between AD and ACLF. This distinction is not possible with the albumin concentration [16].

Mann-Whitney-test vs. AD (* p<0.0001; # p=0.0001)

Principal component analysis (PCA)

A PCA was applied to five parameters of the albumin function test. The first main component (PC1) already covers 89% of the total variance in patients with AD and 86% in ACLF and was therefore selected to represent the remaining albumin function.

In a multiple linear regression both tAlb (total albumin concentration) and eAlb (effective albumin concentration) and the MELD score were shown as independent predictive values for the remaining albumin function, described by PC1 [16].

	Standardized Coefficient	P Value
AD (n=241)		
tAlb (g/dl)	0,386	<0,001
eAlb (g/dl)	0,259	0,001
MELD score	-0,165	0,002
ACLF (n=78)		
tAlb (g/dl)	-	-
eAlb (g/dl)	0,350	0,001
MELD score	-0,311	0,003

Prognosis of development of ACLF

The CLIF-C AD score describes the risk of patient with AD to develop an ACLF.
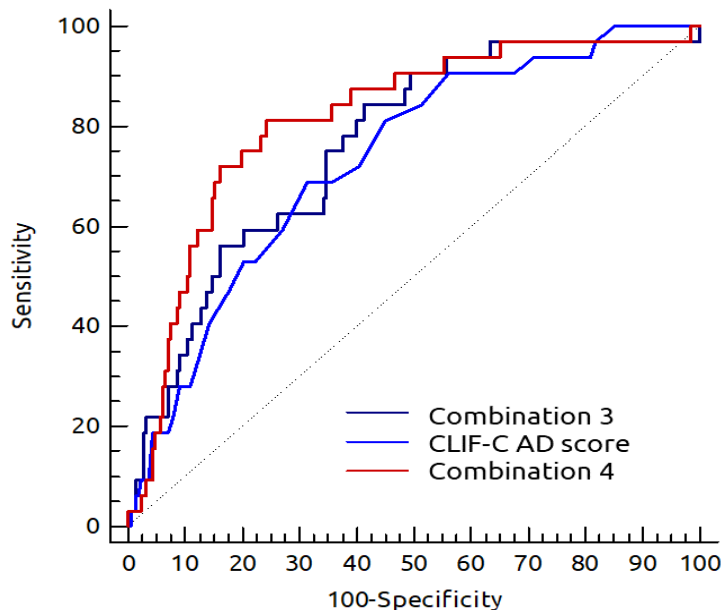
Further analyzes of these study data showed that a combination of parameters of the albumin – functionality-test had the same diagnostic performance (AUC 0.76 [0.70-0.81]) as the CLIF-C AD score (0.73 [0.67-0.79]).

A combination of both could significantly improve the diagnostic performance of the CLIF-C AD score alone (0.81 [0.76-0.86]). (Manuscript in progress)

Prognosis of 1-year-survival by use of parameter of albumin-functionality-test

Patients with a parameter (C5B) greater than 0.57% (red line) showed a significantly better survival than patients with a value less than 0.57% (blue line).

365 days survival time from admission
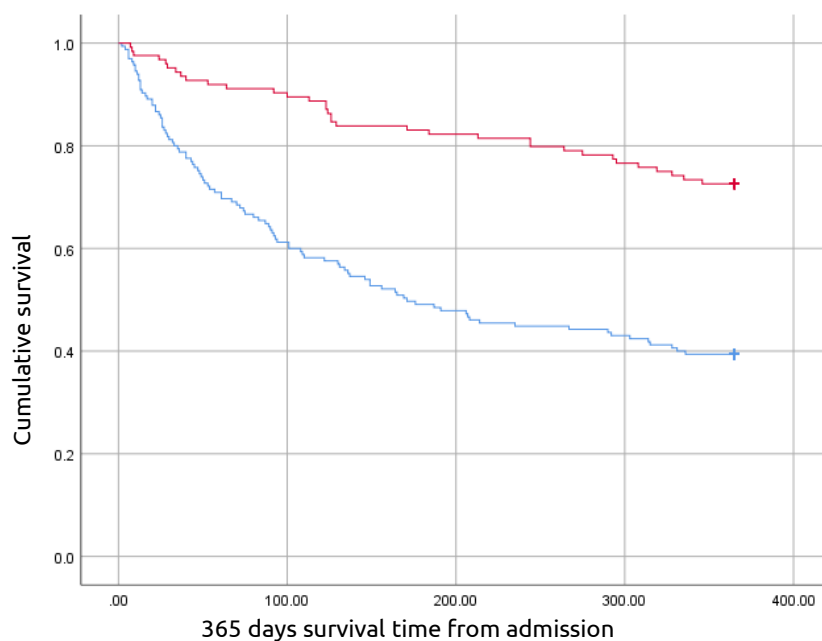
A Kaplan-Meier survival analysis of hospitalized patients (AD and ACLF) revealed a significant difference in terms of survival rate based on attachment efficiency (P = 0.001).

One parameter of the albumin function test (C5B) was able to improve this significantly (P <0.001). (Manuscript in progress)

DTE in patients with sepsis or SIRS

In a retrospective and masked pilot study the transport properties of albumin of patients in an intensive care unit were investigated (unpublished data).

Five patients developed a SIRS* (Systemic Inflammatory Response Syndrome) and five a sepsis.

The transport parameter DTE in patients with sepsis or SIRS was compared to ICU patients without sepsis or SIRS (labeled as normal).

normal (n=11), SIRS (n=5), sepsis (n=5)
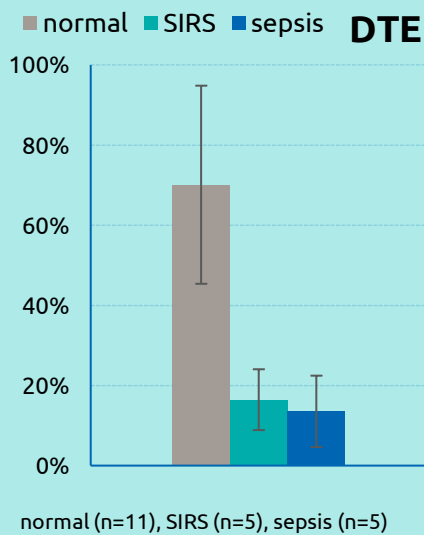
With values of less than 20% the detoxification efficiency is distinctly reduced in both groups in comparison to ICU patients without sepsis or SIRS .

* Definition before Sepsis-3

Time course of DTE

From these patients blood was taken at the day of entry and 60, 36 and 12 hours before diagnosis by a standard method.

There are significant differences (p = 0.009, u-test) in the progress of the DTE between sepsis and SIRS.

While the DTE values of the patients with SIRS remain almost constant, the values of the patients with sepsis decrease over time.

So it might be possible, to predict the progress of a sepsis with high probability.
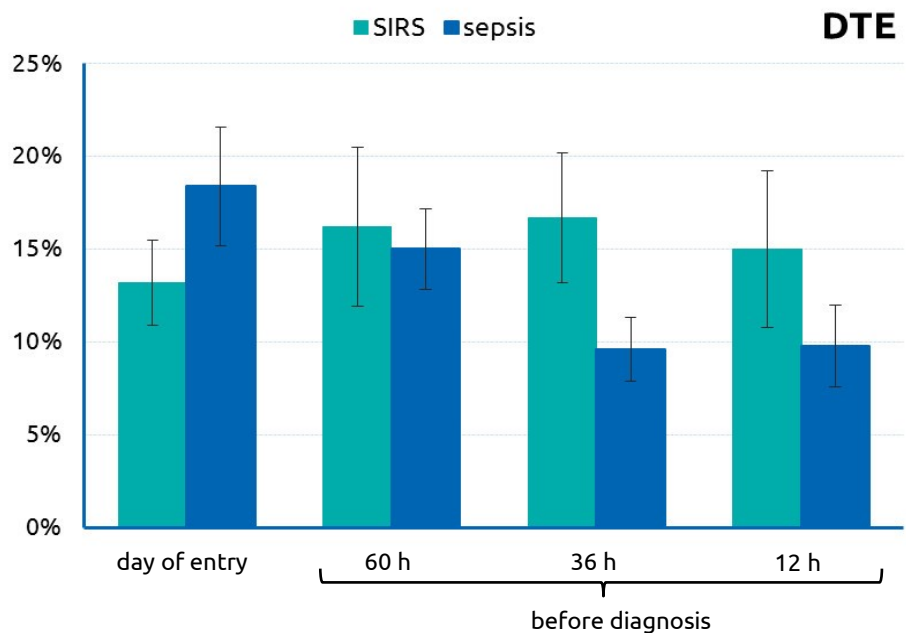

This can be realized by daily determination of the albumin transport parameters initiated from day 1 of the patient monitoring.

Hence a tendency will be timely recognized, and systemic measures could be initiated earlier.

These first findings will have to be verified in a study with larger numbers of patients [university of Rostock and IZI Fraunhofer; DRKS00025079.
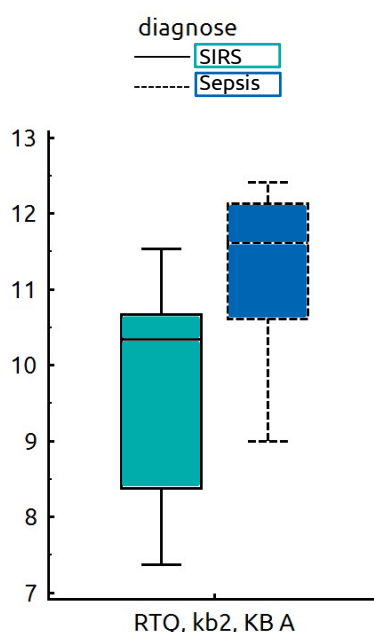
Another pilot study examined patients with sepsis or SIRS at the time of diagnosis. Other clinical parameters were compared with those of the albumin function test in order to find out whether the albumin function test had an equivalent or even better diagnostic quality in the separation of sepsis and SIRS.

■ SIRS (n=10) ■ Sepsis (n=10)

■ SIRS (n=10) ■ Sepsis (n=10)
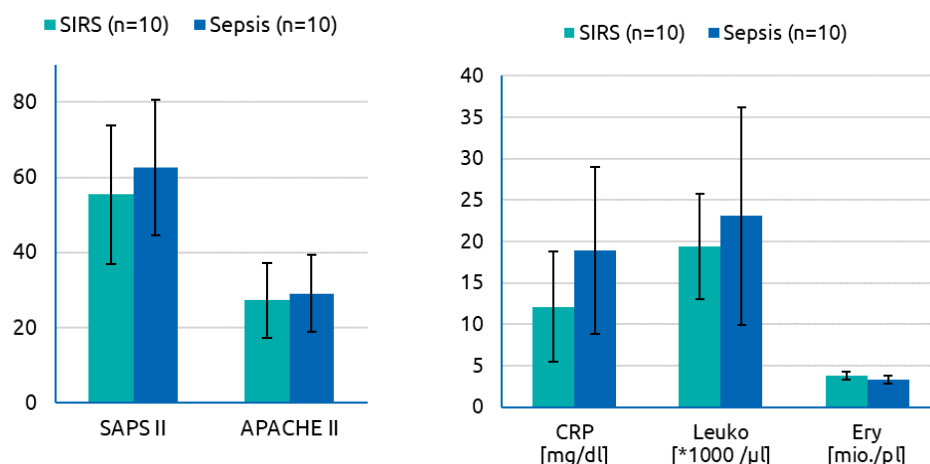
As shown in the figures and the table, SAPS II, APACHE II and the number of leukocytes show almost the same values for both groups. The CRP, number of erythrocytes, BE, DTE and KB A show differences which, however, do not reach the significance.

Only the RTQ and kb2 show significant differences.

For patients with sepsis or SIRS other clinical parameters like:
- SAPS II (Simplified Acute Physiology Score)
- APACHE II (Acute Physiology And Chronic Health Evaluation)
- C-reactive protein
- Procalcitonin
- blood count

were also available.

As procalcitonin was available only for 50% of the patients, it was not used for statistics.

	u-test			u-test
SAPS II	0.473		**BE**	0.082
APACHE II	0.650		**RTQ**	**0.049**
CRP	0.141		**DTE**	0.131
leuko	0.821		**kb2**	**0.049**
ery	0.059		**KB A**	0.096

significant P<0.05

Conclusions

Although only in an initial feasibility study with small group sizes, parameters of the albumin-functionality-test have shown the potential to be useful parameters in patients with sepsis and SIRS and in the differentiation between these two groups.

Further studies are needed to verify these findings.

Using logistic regression, a parameter combination of RTQ, kb2 and KB A was found, which can significantly differentiate between patients with sepsis and SIRS and this better than the individual parameters (P = 0.023).

diagnose
—— SIRS
-------- Sepsis

RTQ, kb2, KB A

Sepsis

Conclusions

In patients with sepsis or SIRS the determination of the detoxification efficiency from the albumin-functionality-test could be used for a disease progression monitoring.

In order to validate this and the previous data, further studies were initiated.

With the Rostock University Hospital and IZI Fraunhofer (DRKS00025079), patients with sepsis, septic shock and ITS patients without septic diseases are first compared, followed by long-term observation.

CytoSorbents Europe GmbH is carrying out a study on the use of their CytoSorb ® adsorber. The albumin-functionality-test is used as a diagnostic procedure.

In all studies, the parameters of the albumin-functionality-test are not only compared with the established parameters, but also combinations of parameters are always examined. The aim is to achieve the greatest possible diagnostic performance.

Long term monitoring

In a prospective study with patients of an intensive care unit blood samples were collected from the day of entry until leaving ICU and transfer to another department.
Two different patients are exemplarily depicted.
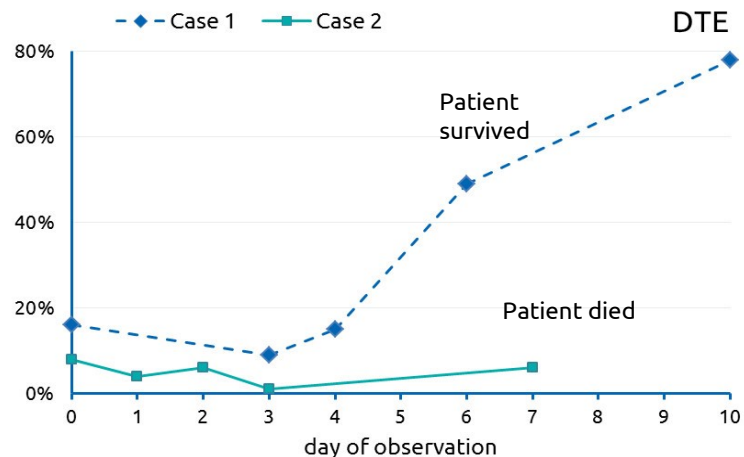
The time course of DTE reflects the clinical conditions of the patients.

Pre-study intensive care medicine

In the above-mentioned study with University of Rostock and Fraunhofer IZI ten patients of intensive care medicine without any suspicion for sepsis or septic shock were compared to ten patients with sepsis and six patients with septic shock.
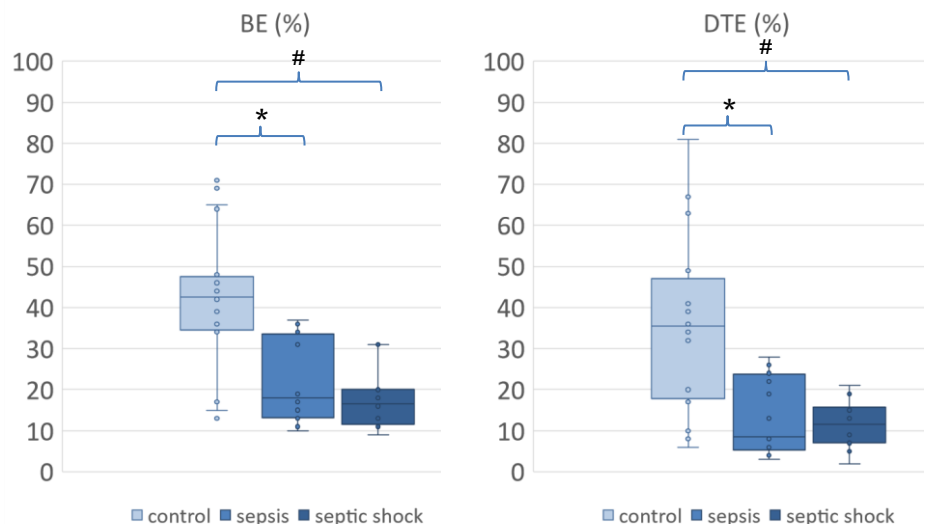

Mann-Whitney-test (independent samples) *P<0,01, # P<0,03

All patients show strongly reduced transport parameters in comparison to healthy individuals (100%). Binding efficiency as well as detoxification efficiency show further significant reduction in patients with sepsis or septic shock in comparison to patients without these complications [17].

Patients with kidney disease

Before hemodialysis treatment (n=58), albumin binding and detoxification efficiency were substantially below those in healthy individuals (100%) and even declined after dialysis treatment [18].
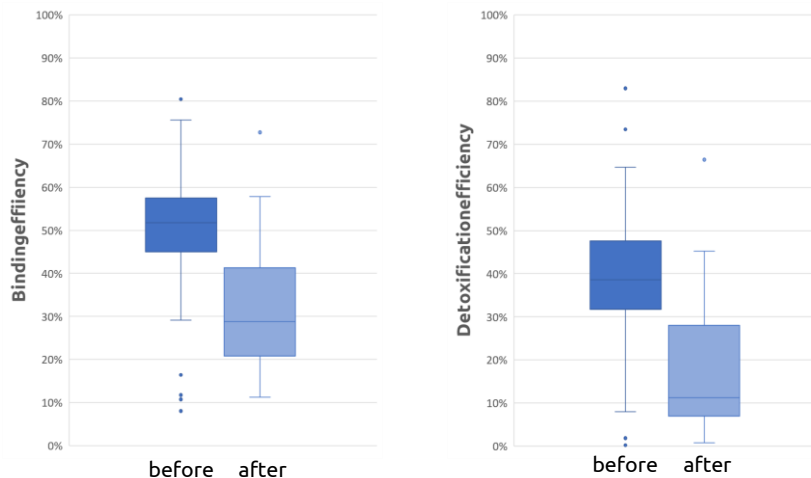
This correlates strongly with concentration of non-esterified fatty acids (NEFA), which increase after treatment. This is associated with heparin anticoagulation.

In cooperation with University Hospital Essen samples of patients before and after hemodialysis treatment were analyzed to determine the influence of treatment on albumin functionality.

Further investigations regarding type of anticoagulation during hemodialysis and other therapy options including kidney transplantation are currently in progress.

Filtering of albumin

Commercial human albumin solutions (HSA) show reduced transport parameters before filtering.

After a filtration of the albumin solution with the Hepalbin™ – Adsorber (Albutec GmbH), the concentration of stabilizers could be reduced.

First results showed strikingly improved detoxification effi-ciency and binding efficiency (BE) of the filtered albumin.

albumin solutions were diluted to 45 g/l
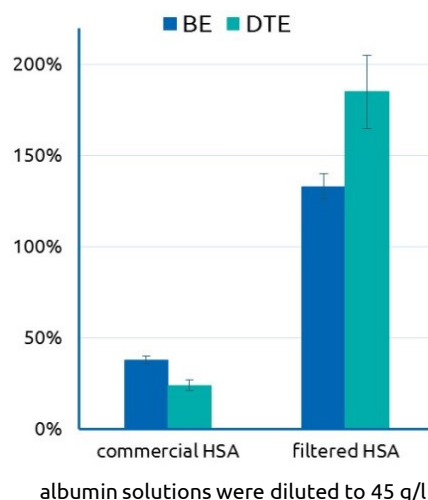
These parameters are regenerated to physiological values and above.

Conclusions

Commercial albumin solutions used for patients with various diseases like liver failure, hepatorenal syndrome or ascites, might be applied with caution.

Not only these albumin solutions are not able to bind as much toxins as expected in the patient blood, but also add unwanted molecules (stabi-lizers) to a weakened organism.

References

1. Peters TJ (1995) All about Albumin: Biochemistry, Genetics, and Medical Applications Academic Press, New York
2. Bhattacharya AA, Grune T, Curry S (2000) Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. J Mol Biol 303: 721-732
3. Simard JR, Zunszain PA, Ha CE et al. (2005) Locating high-affinity fatty acid-binding sites on albumin by x-ray crystallography and NMR spectroscopy. PNAS 102: 17958-17963
4. Kawashima Y, Fukutomi T, Tomonaga T et al. (2010) High-yield peptide-extraction method for the discovery of subnanomolar biomarkers from small serum samples. J Proteome Res 9: 1694-1705
5. Lowenthal MS, Mehta AI, Frogale K et al. (2005) Analysis of albumin-associated peptides and proteins from ovarian cancer patients. Clin Chem 51: 1933-1945
6. Mehta AI, Ross S, Lowenthal MS, Fusaro V et al. (2003) Biomarker amplification by serum carrier protein binding. Dis Markers 19: 1-10
7. Taboada P, Barbosa S., Emilio Castro et al. (2007) Effect of solvation on the structure conformation of human serum albumin in aqueous–alcohol mixed solvents. Chemical Physics 340: 59-68
8. Reed RG, Burrington CM (1989) The albumin receptor effect may be due to a surface-induced conformational change in albumin. J Biol Chem 264: 9867-9872
9. Xu Y, Shen Z, Wiper DW; et al. (1998) Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. JAMA 280(8): 719-723
10. Seidel P, Gurachevsky A, Muravsky V, Schnurr K et al. (2005) Recognition of malignant processes with neural nets from ESR spectra of serum albumin. Z Med Phys 15: 265-272
11. Kazmierczak SC, Gurachevsky A, Matthes G et al. (2006) Electron spin resonance spectroscopy of serum albumin: a novel new test for cancer diagnosis and monitoring. Clin Chem 52: 2129-2134
12. Gurachevsky A, Kazmierczak SC, Jörres A et al. (2008) Application of spin label electron paramagnetic resonance in the diagnosis and prognosis of cancer and sepsis. Clin Chem Lab Med. 46(9): 1203-10
13. Gelos M, Hinderberger D, Welsing E, Belting J, Schnurr K, et al. (2010) Analysis of albumin fatty acid binding capacity in patients with benign and malignant colorectal diseases using electron spin resonance (ESR) spectroscopy. Int J Colorectal Dis 25: 119-127
14. Moergel M, Kammerer PW, Schnurr K et al. (2012) Spin electron paramagnetic resonance of albumin for diagnosis of oral squamous cell carcinoma (OSCC). Clin Oral Investig 16: 1529-1533
15. Jalan R, Schnurr K, Mookerjee RP et al. (2009) Alterations in the functional capacity of albumin in patients with decompensated cirrhosis is associated with increased mortality. Hepatology 50: 555-564
16. Baldassarre M, Naldi M, Zaccherini G, et al. (2021) Determination of effective albumin in patients with decompensated cirrhosis: Clinical and prognostic implications. Hepatology doi: 10.1002/hep.31798
17. Klinkmann G, Waterstradt K, Klammt S, Schnurr K, Schewe JC et al. (2023) Exploring Albumin Functionality Assays: A Pilot Study on Sepsis Evaluation in Intensive Care Medicine. Int J Mol Sci. 24(16):12551.
18. Boss K, Waterstradt K, Schnurr K, Paar M, Stolpe S et al. (2023) Binding and detoxification efficiency of albumin decline after haemodialysis. Nephrol Dial Transplant. Aug 9 Online ahead of print.

MedInnovation
Advanced Albumin Research