Identification of Patients with Pancreatic Cancer by Electron Paramagnetic Resonance Spectroscopy of Fatty Acid Binding to Human Serum Albumin

Haleh H. Haeri, Jörg Tomaszewski, Bettina Phytides, Heike Schimm, Gabriela Möslein, Marco Niedergethmann, Dariush Hinderberger,* and Marcos Gelos*

Cite This: *ACS Pharmacol. Transl. Sci.* 2020, 3, 1188–1198

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ABSTRACT: An effective biological marker for pancreatic adenocarcinoma (PAC) is not available so far. Here, we investigate how electron paramagnetic resonance (EPR) spectroscopy of spin-labeled fatty acid (FA) molecules binding to human serum albumin (HSA) in human serum is a suitable method for the identification of patients with PAC through detection of PAC-induced changes of FA binding to albumin. The functionality of HSA to bind FA is investigated in serum samples of 35 patients with PAC, 26 patients with benign pancreatic tumors (BPD), and 24 healthy individuals by continuous wave (CW) EPR spectroscopy by simply dissolving 16-DOXYL stearic acid as spin-labeled FA. It is found that FA binding to HSA in PAC is significantly modified when compared with healthy and BPD individuals. The PAC group could best be discriminated from the healthy group based on EPR characteristics at the loading ratio of 1:4 (HSA:FA), while patients with PAC and BPD are distinguishable at a loading ratio of 1:6. Using nanoscale distance measurements through double electron–electron resonance (DEER), it is found that the distribution of FAs in the HSA of one PAC patient is similar to that of FAs in healthy individuals. Combining all EPR spectroscopic data, this leads to a tentative molecular interpretation of only small changes in hydration at the protein's surface as origin of the detectable characteristics for PAC patients. Thus, EPR of FA/HSA binding is a simple and promising tool for clinical detection of patients with PAC and needs to be tested with larger ensembles of different patient groups.

KEYWORDS: albumin, electron spin/paramagnetic resonance spectroscopy, ESR/EPR, DEER, pancreatic cancer, pancreatic adenocarcinoma, IPMN, precancerous pancreatic lesion, tumor marker, biological marker

INTRODUCTION

Human serum albumin (HSA) is a 585 amino acid (~66.5 kDa) serum protein and the main transporter of a variety of peptides and of water-insoluble, hydrophobic compounds, or amphiphilic molecules like long-chain fatty acids (FAs) in human serum.¹ Seven long-chain FA binding sites of HSA have been described so far² with different affinities toward binding FAs (see Figure 1A). These binding sites play a role in the transport of hydrophobic substances such as endotoxins.^{3,4} Binding or even weak association of metabolites, drugs, and toxins or post-translational chemical modifications can lead to changes of the highly dynamic HSA solution conformation.^{5–8} As a result, the transport capacity of albumin depends on its ability to (allosterically) bind metabolites, the capacity of binding sites, intensity of ligand binding, and the degree of dissociation of bound ligands toward their target cells.^{9,10} When proteins released from tumor cells bind to HSA, changes in the dynamic albumin structure and transport capacity can be induced.^{9,11} As a consequence, the functionality of HSA to bind and transport FAs is modified. Since the seven FA binding

sites are distributed throughout the complete three-dimensional protein structure, alterations of (secondary) structural elements likely affect at least one of the binding sites, e.g., by changing their binding affinities or their relative orientations inside the protein. These modifications can then be detected by studying the FA binding to HSA using electron paramagnetic resonance (EPR) spectroscopy.^{2,5} Obeying spin physics similar to those known from nuclear magnetic resonance techniques (like MRI), in EPR one detects unpaired electron spins in radicals that in case of HSA are introduced artificially by adding spin-labeled stearic acid (16-DOXYL stearic acid, 16-DSA, see Figure 1B), i.e., a stearic acid with a stable nitroxide radical in position 16 of the hydrocarbon chain

Received: August 19, 2020

Published: October 2, 2020

Figure 1. (A) HSA crystal structure (RCSB Protein Databank (PDB): 1e7i) with seven stearic acid molecules.¹⁸ The oxygen atoms of the FA carboxylic acid head groups are shown in red. (B) Chemical structure of the EPR active spin-labeled stearic acid, 16-DSA.

(for simplicity, we often refer to the spin-labeled FA as “FA”). An approach combining standard continuous wave (CW) EPR spectroscopy with the pulse EPR method “double electron–electron resonance” (DEER) has in recent years been developed into a research platform for the determination of functional characteristics of plasma proteins and in particular albumin.^{2,5–8,12–15} Using CW-EPR spectroscopy, the binding characteristics of FA to albumin thus allow deep insights into HSA function and its ligand binding and release properties.^{8,14} These include conformational changes of HSA leading to less tightly bound FA, the number of high-affinity FA binding sites of HSA and hence the occupation of binding sites by endotoxins, metabolites, or peptide fragments. There have been several initial attempts to develop EPR-based tests for diseases,^{16,17} and this variability in HSA structure has been observed in patients with malignant colorectal diseases.¹⁵

As we have recently shown,¹² one can base the rigorous, quantitative understanding of FA/HSA binding on EPR spectral parameters and their differences when comparing commercially available and purified HSA in buffered aqueous solution with that in blood serum of healthy individuals. We have investigated serum samples of 24 healthy individuals and found that two spectral parameters reveal deviations from corresponding values for buffered solutions of purified HSA. Such variations reflect the impact of the physiological serum, of which albumin is only the major component (by far) on FAs. Remarkably, a parameter range for healthy individuals was found that still is rather narrow. Furthermore, negative cooperativity was observed for most of the samples in the healthy adult group.

Pancreatic adenocarcinoma (PAC) is associated with a high mortality and poor prognosis, as indicated in the overall 5 year survival of approximately 10%.¹⁹ Patients eligible for curative surgery have a 5 year survival rate of 25%,^{20,21} and only 10–15% of patients with PAC can be healed.²² These numbers

could be improved if diagnosis of patients with PAC was possible at an earlier stage of disease, which would likely increase the overall survival rate of PAC patients.^{23,24} Despite the apparent lack of clinically detectable symptoms, a rather large “window of opportunity” is suggested to exist in which PAC maybe detected at an early enough stage for successful intervention.²⁵ So far, diagnosis of PAC is based on computed tomography (CT), magnetic resonance imaging (MRI), or endoscopic techniques. In unclear situations, even a surgical intervention has to be considered to obtain a diagnosis.²⁶

Therefore, a screening tool, e.g., in form of detecting a biological marker, would be of great significance to detect PAC early and/or to discriminate PAC from benign pancreatic lesions (hereafter named as benign pancreatic disease, BPD) such as chronic pancreatitis (CP), intraductal papillary mucinous neoplasms (IPMN), and other benign lesions.^{27,28} Also, screening patients having gone through treatment for PAC or even persons with a family background of PAC could be viable options in the case that a reliable and simple biomarker was available. CA 19–9, a carbohydrate antigen, is the only biomarker routinely applied in the diagnostic management and follow up of PAC. However, CA 19–9 may also be elevated in benign pancreatic diseases, e.g. inflammations or benign cases of biliary obstruction,²⁹ and has a sensitivity and specificity of 85% for detecting pancreatic cancer.³⁰

The long-term translational science goal in our studies is to use the above-described, simple CW-EPR spectroscopic method as a screening tool for initial detection of diseases which are hard to detect (if at all). In principle, this is possible for all diseases that directly or indirectly affect FAs binding to HSA. Besides the mere detection of disease states, importantly one may continue to understand on which kinds of structural modifications or interactions this measurable state is based. Since PAC and especially the differentiation of PAC from BPD is currently not possible in a reliable manner, with the present work we aim at exploring the capabilities of EPR spectroscopy to discriminate between patients with pancreatic cancer from healthy individuals and patients suffering from benign pancreatic entities by analyzing their FA binding patterns to HSA. This is the first step in translating this biophysical characterization method into clinical use.

RESULTS

EPR Spectroscopic Parameters. A detailed explanation of the EPR-spectroscopic approach is given in ref 12. Here, we give a short introduction on which spectroscopically available parameters were analyzed. Of the main EPR characteristics employed, we study the conformational flexibility of HSA in terms of the rotational correlation times (usually denoted τ_c in nanoseconds, ns) of the bound FAs. As it has been shown before,¹² τ_c has the capacity to give a quantitative measure for HSA functionality and conformational changes.^{2,14} Previously, we have shown that two types of FA binding can be discriminated through their effect on the rotational motion of the FA (τ_c). “Strong immobilization” binding sites (hereafter called (A) sites) immobilize bound FA strongly, and “intermediate immobilization” binding sites (B) immobilize FA less strongly ($\tau_c < 5$ ns). As a second EPR-based property, we used the apparent hyperfine coupling ($2A'_{zz}$) which is defined as the distance between the two outermost lines of the spectrum and is a hyperfine coupling component along the molecular frame z -axis.² This property is correlated to local

molecular polarity in the immediate environment of the FA (the higher the polarity, the larger A'_{zz}). Polarity variations of bound ligands can be directly correlated with the presence of more or less waterlike media, which could be attributed to conformational changes of HSA.² Also for A'_{zz} , we found sensitivity toward the binding site types (A) and (B).¹² Both EPR characteristics are additionally determined by the percent of their contribution to the overall spectral simulation. Among the examined ratios of HSA to FAs (1:0.5 to 1:7 in steps of 0.5 equiv of FAs), the 1:4 ratio shows most specific changes.¹² As an example, Figure 2 shows the comparison between the samples of an individual diagnosed with PAC (upper spectrum) and a healthy one (lower spectrum) at the loading ratio of 1:4.

The experimental spectra are given with the corresponding simulations. Obviously there is no large difference between

Figure 2. CW-EPR spectra (black) and simulations (red, shifted upward for clarity) of spin-labeled FA (16-DSA) added at a HSA:FA loading ratio of 1:4 to blood serum of two individuals, one diagnosed with PAC and one healthy individual of the control group. For a reliable analysis of the data, one needs to consider variations in hyperfine coupling as indicated by the splitting denoted $2A'_{zz}$, the rotational correlation times (complex spectral dependence) and population of different groups of binding sites (A) and (B).

$2A'_{zz}$ and the amount of nonbound (free) FA. However, the spectra show distinct line shapes and dynamic behavior which result in different rotational motions as characterized by the rotational correlation times. Additionally, the population of sites (A) and (B) are significantly different. Hence, we monitor both of these EPR characteristics, $2A'_{zz}$ and τ_c .

When comparing the results of the two EPR characteristics for different patient groups, significant differences concerning the measured τ_c or A'_{zz} values become evident for specific ratios of HSA to FA. We report corresponding values for 1:4 and 1:6 of HSA:16-DSA which display the most pronounced differences. These values are provided for both binding sites, strongly immobilized (A) and intermediately immobilized (B), respectively, and are displayed in Table 1. The largest differences were observed for loading ratios of 1:4 and 1:6.¹² The summary of the numerical values obtained by simulations are given in Table 1. Also, the graphical form of the obtained statistics on these data are drawn in boxplots. As an example, the boxplot of the hyperfine coupling values for all studied patient groups, PAC, BPD, and healthy individuals, discriminated by binding sites (A) or (B), at a 1:4 loading ratio is given in Figure 3. The detailed plots can be found in Figures S1–S3.

When plotting $2A'_{zz}$ against the rotational correlation times τ_c at both ratios, one finds that the corresponding values for binding sites (A) and (B) are fully separated and distinguishable from each other for all three groups. The binding sites (A) have larger $2A'_{zz}$ than those of sites (B), shifted by 8–9 MHz and the corresponding correlation time diminishes from ~ 13 to ~ 2.5 ns by going from sites (A) to (B) (Figure 4 and S4–S6).

Considering the control group as biologically relevant reference, the difference between hyperfine couplings and correlation times of all groups at the loading ratio 1:4 for binding sites (A) are shown in Figure 5. As there are no large differences between the BPD samples and control group at this loading ratio, in the following we only compare the obtained data between PAC–control and BPD–PAC groups. Another important and potentially informative parameter is the amount of free (non-HSA-bound) FAs. In all spectral analyses, we find this number to be similar to the values shown by the healthy group samples. The amount of free FAs increases when raising the HSA:FA ratio consecutively from 1:3 (where a free component appears at all for the first time) to 1:7. This is expected and well understood (see refs 5 and 12), as the binding process is dynamic. The median values of the free component of the spectra are well below or close to one percent in all samples and do not differ significantly from the values for the other groups.¹² As an example, the relative contribution of the free FA component at different loading ratios for PAC samples is given in Figure S7.

PAC versus Control Groups at Loading Ratio 1:4. The PAC patient group samples could be well-distinguished from healthy control samples by clear differences of their EPR characteristics for the (A) binding sites. In the PAC group samples, FA experience a more polar medium (having ~ 4 MHz larger $2A'_{zz}$) and are faster in their rotational dynamics by ~ 4 ns. They also show a higher population in the strong immobilization binding sites (A), as compared to the control groups. The two groups are distinguishable from each other by both their EPR characteristics for the (A) sites, while for the (B) sites the only diagnostic variable is the rotational correlation time (Figure 5 and Table S2).

Table 1. Median Values of the Simulation Parameters ($2A'_{zz}$, τ_c) for All Measured Blood Samples in This Study and for Purified HSA^a

	HSA:16-DSA ratio	($2A'_{zz}$, τ_c (A)) in MHz and ns, respectively	($2A'_{zz}$, τ_c (B)) in MHz and ns, respectively
PAC	1:4	(99.20 (1.62), 9.03 (2.32))	(89.60 (1.22), 2.23 (0.78))
	1:6	(99.0 (1.68), 8.11 (2.35))	(90.7 (1.92), 2.46 (0.89))
BPD	1:4	(96.55 (0.99), 13.49 (1.09))	(88.60 (1.10), 2.86 (0.53))
	1:6	(96.05 (0.67), 13.73 (1.22))	(88.60 (1.06), 3.14 (0.66))
control	1:4	(95.70 (2.60), 13.43 (2.70))	(89.1 (0.00), 4.22 (0.80))
	1:6	(97.10 (0.80), 12.60 (0.60))	(91.50 (0.90), 3.67 (0.20))
purified HSA	1:4	(97.10, 12.80)	(91.50, 3.20)
	1:6	(98.80, 8.60)	(91.50, 2.70)

^aBinding sites with strong and intermediate immobilization are denoted by (A) and (B), respectively. The MAD (median absolute deviation) is given in parentheses. Data for purified HSA are from ref 12.

Figure 3. Boxplot of the hyperfine couplings ($2A'_{zz}$) for FAs of the different patient groups in the two types of binding sites (A) and (B) of HSA at a loading ratio 1:4. The red lines and corresponding values are the medians in each group. As can be seen, the value for binding sites (A) of the PAC group strongly deviates from the corresponding values of the healthy control group, while for the binding sites (B) the values are almost indistinguishable at this loading ratio.

PAC versus BPD Groups at Loading Ratio 1:4. For the PAC samples, when compared to the BPD samples, the FAs sense a more polar immediate environment and display faster dynamics. Moreover, in the PAC samples the binding sites of type (A) are more populated than the ones of the BPD samples by about 2.5%. More precisely, the median of the contributions of the binding sites (A) are obtained as 81.6% for PAC samples, while this value for BPD group is about 79.3%. A list of contributions of each of the binding sites (A) and (B) to their total simulated spectrum of the two groups are given in Tables S3 and S4. The corresponding values for the control group are collected in Table S5.

Taken together, one can conclude that the binding sites in HSA of PAC patients show a propensity to be more exposed to the blood solution and/or bind more hydrophilic ligands. Confirmed by the Welch-Satterthwaite version of the *t* test, they could be well-discriminated from each other using both $2A'_{zz}$ and τ_c ($\sim 10^{-7}$ and 10^{-8}). The graphical presentation is shown in Figure 6. The binding sites (B) of the PAC and BPD groups show very similar trends. They are found to be

Figure 4. Correlation between spectral features $2A'_{zz}$ versus τ_c are shown at a loading ratio of 1:4. Differences in dynamics are resolved for different binding sites such that the two motional regimes of two binding sites are visibly separated. The size of the points is scaled to the quantity (occurrence) of the respective value (the larger, the more simulations gave these values).

indistinguishable from each other at this loading ratio (Table S2).

BPD versus Control Groups at Loading Ratio 1:6. At higher loading ratios, we find different binding-site-dependent sensitivity in the spectroscopic parameters. For the (A) sites, the samples of two groups could be best separated from each other based on their correlation times, while at sites (B) it is the hyperfine coupling which could be used as a marker (Table S2).

PAC versus Control Groups at Loading Ratio 1:6. For the PAC samples, two distinct rotational behaviors are observed for FA in binding sites (A) at a 1:6 loading ratio. These two rotational FA species are identical to those found for PAC samples at a 1:4 loading ratio and both are faster than those of the control group (by ~ 5 and ~ 2 ns, respectively, Figure 7). The second species also features smaller $2A'_{zz}$ and thus has behavior rather similar to that of the healthy control samples. There is a clear distinction between values for PAC and control group samples for their binding sites (A). At binding sites (B), these two groups behave differently from each other in terms of their correlation times ($p \sim 10^{-3}$).

PAC versus BPD Groups at Loading Ratio 1:6. The statistical reliability difference test indicated that the two diseased groups could be best separated from each other based

Figure 5. Bar charts of the $\delta 2A'_{zz}$ (panel A) and $\delta \tau_c$ (panel B) of the studied groups in their strong immobilization binding sites (A) at loading ratio of 1:4 considering the control group as the reference. The PAC group can be discerned from the healthy control and BPD groups in both EPR characteristics.

Figure 6. Discrimination of PAC (values from the PAC patient group shown as red circles) and BPD (black circles) patient groups as observable in a scatter dot plot. These two groups are distinguishable based on their EPR characteristics at their strong immobilization binding site (A) at the 1:4 loading ratio. The median contribution of binding site (A) for all samples in the PAC and BPD groups are 81.7 and 79.3%, respectively.

Figure 7. Scatter dot plots of $2A'_{zz}$ versus τ_c for all groups at a loading ratio 1:6 are shown: healthy control (green) BPD (black) and PAC (red). The PAC samples again indicate existence of two distinct FA species, one being faster (~ 7 ns, 19 samples out of 35, the so-called “set 1”) with larger $2A'_{zz}$, while the other is slower (~ 11 ns) and has a smaller $2A'_{zz}$ that is rather similar in behavior to the control group samples. The set 1 samples at this loading ratio have similarities with the PAC samples at the lower loading ratio 1:4.

on both EPR characteristics of the binding sites (A) (see Table S2).

DISCUSSION

Consideration on Clinical Application. Screening programs in high-risk populations are expected to yield more patients with pancreatic cancer at an early stage.³¹ About 80% of patients with PAC present in an advanced stage of disease without chance of curative treatment. Thus, only a limited number of patients with early stage disease are included in studies of early detection markers.³²

PAC arises from noninvasive precursor lesions, which are surgically curable.²⁵ One of the major source of such precursor lesions are IPMNs, representing 50% of resected pancreatic cysts.³³ One third of them harbor invasive adenocarcinomas.³⁴ These facts underline the difficult clinical problem to differentiate between a benign and malignant pancreatic tumor. Furthermore, progression of an IPMN or MCN to a carcinoma takes years to decades. Hence, there is a certain time range to detect potentially curable lesions of the pancreas before progression to carcinoma.²⁵ Patients with surgically treated stage I PAC have a median survival time of 78 months and a 5 year survival rate of 58%.^{21,35} Therefore, the effort has to be undertaken to achieve diagnosis at an early stage in hope for a reduction in mortality. Ideally, this should occur at a precancerous stage, as in colorectal carcinoma.³⁶ It would thus be helpful to develop a test to detect curable lesions and distinguish lesions with a high probability of developing to cancer from lesions with no or little risk.²⁵ Many studies have examined potential novel biomarkers to identify early pancreatic neoplasia show potential in the role of detection of PAC: circulating tumor cells, proteins, mucins, metabolites, cell-free DNA (cfDNA), noncoding RNA, and exosomes.^{37,38} However, none of them is established as reliable marker.³⁰

HSA is the main serum transport protein. One of its tasks consists of binding peptides, which are released under a variety

of biological situations.¹ It is known that malignant diseases may influence concentration as well as function of HSA.^{10,39} In the present work, we demonstrate the ability of CW-EPR spectroscopy on a simple-to-use and cheap (that is, with prices comparable to conventional ultrasound devices) bench-top spectrometer to analyze blood samples and address a highly prevalent clinical question.

This is the first study examining EPR spectroscopic data of spin-labeled FA binding to HSA in a panel of patients with pancreatic pathologies. The main purpose is to evaluate the potential capabilities of EPR spectroscopy as a detection tool for PAC versus benign pancreatic diseases and healthy controls, i.e., a proof-of-concept test toward translating this method to more routine clinical use and through larger patient numbers. The present examination is built on the established analysis of FA binding to HSA by EPR spectroscopy in healthy individuals and commercially available albumin.¹² The differences between the studied groups could be observed in terms of rotational correlation times and changes of hyperfine coupling of FA bound components as the main variables. The physicochemical importance of this study is that it is performed under conditions as precisely controlled as standardized HSA:FA ratios. Another strength of the applied method is its capability to display changes in HSA through the effects on fatty acid binding, a well-understood and well-characterized process.

In patients with pancreatic adenocarcinoma, data derived from EPR spectroscopic analysis of 16-DSA/HSA show significantly different results than in those patients with benign pancreatic lesions, as well as from those in healthy individuals. From the data presented in the “Results” section, the differences can be classified as the following: (1) As a general trend, HSA-bound FAs have faster dynamics in PAC samples compared to those from patients with BPD and with healthy individuals, while for BPDs this difference to healthy individuals is marginal. (2) HSA from diseased patients, i.e., BPD and PAC, takes up FA differently than that of the healthy control group, as also observed by the $2A'_{zz}$ values and to a lesser extent by the percent contributions (see Table S2, Figures S4 and S6). This effect is observed for PACs at lower loading ratios (1:4), while for BPDs, it manifests itself mainly at higher loading ratios of (1:6). In fact, at a 1:4 ratio, the BPD samples have a rather similar behavior as the control samples. (3) Between the two binding sites, it is the strongly immobilized one (A) which is mostly affected in PAC patients. One can differentiate between PAC versus BPD or healthy individuals best at (A) sites of a 1:4 loading ratio, while BPD patients are distinguishable from healthy individuals at their more loosely bound site (B) and at the 1:6 loading ratio. (4) There is a widespread scattering range of data for the healthy control group, but a rather narrow parameter range is covered for PAC patient and BPD patient samples.

Concerning the potential role as detection marker, one can conclude from the present results that EPR spectroscopy may play a role in diagnosis of pancreatic cancer. Patients with benign pancreatic diseases showed specific alterations in their EPR spectra that made them distinguishable from patients with pancreatic cancer and healthy individuals; in particular, the former distinction is not possible from a biomarker or screening test currently. Importantly, the three groups did not differ significantly in age. If a potential biomarker is examined for early detection, then the control group should reflect the population that is expected to be screened. If

controls were young, then the discrimination capabilities of a biomarker may be overestimated.²⁶ Patients with PAC examined in the present study displayed a remarkably homogeneous behavior in EPR analysis when compared with healthy individuals and the EPR characteristics $2A'_{zz}$ and τ_c allowed a differentiation from patients with BPD. Thus, we see a potential application of EPR spectroscopy not only to distinguish between PAC and healthy individuals but also to discriminate between PAC and benign pancreatic processes.

Further studies with a larger number of patients are necessary to underline and validate these new data. Several questions with regard to clinical screening applications remain, e.g., in distinguishing subgroups within the PAC patient group: Is the EPR spectroscopic approach able to discriminate between patients with low- and high-grade precursor lesions of the pancreas? Moreover, may it help as a screening test to detect adenocarcinomas, e.g., in precursor lesions or chronic pancreatitis, to increase the chances of curation by surgical therapy?

Considerations on the Molecular Origin of the Spectroscopic Differences.

As we could show so far, there are statistically relevant differences in the FA-binding of albumin in the serum of patients with PAC and BPD and of healthy individuals. We could identify the relevant spectroscopic parameters of the bound FAs that reveal the differences in FA binding, which may then pave the way for translation into clinical applicability of this approach. When inspecting the approach beyond the mere discrimination of patients, it may be much more scientifically satisfying to also understand the molecular origins of the recorded and proven differences. As described in the Introduction and throughout the paper, the origins of differences in FA-binding to serum-based albumin between the different patient groups can be very diverse. The albumin crystal structure shown in Figure 1 (cocrystallized with stearic acid), gives a static picture of the FA-binding sites. They are usually situated at the interfaces of groups of secondary structure elements, e.g., between bundles of α -helices. As we have shown before, and building on a large previous body of evidence in biophysical publications on albumin, the solution structure of especially HSA is highly dynamic and the protein gains plasticity which mainly affects the protein surface.^{2,5,14} We have ample experience with the characterization of the effect PTMs^{7,8} and physical changes, e.g., in solvent^{6,40} or concentration and pH,^{41,42} have on the dynamic solution structure as seen in the FA binding.

Using this background knowledge, the spectral changes that are pertinent in the serum samples of PAC and BPD patients and healthy individuals can be interpreted in a molecular, nanoscopic fashion. In particular, FAs in the (A)-binding sites with stronger immobilization show a pattern of larger apparent hyperfine couplings $2A'_{zz}$ with faster rotation (τ_c) at all loading ratios. This indicates that the binding in the (A) sites in HSA of PAC patients on average becomes more loose (faster anisotropic rotational reorientation), while the polarity in the immediate environment of the paramagnetic DOXYL-group seems increased (larger $2A'_{zz}$). Combining both findings, we can deduce that the binding pockets apparently seem slightly less tight for the FAs, and when interpreting increased polarity as increased hydration, the chain ends of the stearic acid derivative (16-DSA has the nitroxide label at position 16 of the stearic acid C-18 chain) seem to be more strongly hydrated. Figures S4 and S6 in addition show that the relative contributions of (A)- and (B)-binding sites to the overall

Figure 8. DEER measurements of 16-DSA added to serum samples taken from two individuals, one healthy (in light blue) and one diagnosed with PAC (in black). The measurements are shown for two loading ratios of 1:2 (series A) and 1:4 (series B) of HSA:16-DSA. From left to right: DEER raw data (left) with applied background of dimensionality 3.75 to account for excluded volume effects (in red, see refs 5 and 49), background-corrected DEER time traces (center), and their corresponding distance distribution (right). The maximum dipolar evolution time was chosen as 1.5 μ s as found by echo decay measurements. Minute differences in short distances could be observed, indicating no large-scale alterations in the distribution of FA-binding sites in HSA from this PAC patient as compared to a healthy individual. Validation of such findings should be achieved by expanding the test ensemble of healthy and diseased groups.

spectra alone can probably not be used to discriminate between the different tested groups, but these can help in spreading the data when plotting them against the rotational dynamics and hyperfine couplings. Hence, the changes from a CW-EPR spectroscopic viewpoint seem to be small but detectable at high loading ratios like 1:4 or 1:6, i.e., when many binding sites are occupied. Due to the wide distribution of FA binding sites in HSA, studying the binding of FAs to HSA by EPR spectroscopy is a method that gives an indirect, “coarse-grained” view of the protein’s conformation. Our finely analyzed combined findings thus indicate that large-scale conformational changes of HSA in PAC patients induced by PAC cannot be reconciled with the rather subtle effects found here for FA-binding behavior to HSA on the molecular level. One may then think of a model in which we assume competitive binding of PAC-induced biomarkers such as peptides or proteins to HSA. In such a picture, these biomarkers would partially occupy FA-binding sites either directly or by inhibiting binding allosterically, yet this model is not in accordance with the findings either, most obviously due to the unchanged spectral contributions of the bound and unbound FAs in all three studied groups, as shown in Figures S4 and S7.

Using the four-pulse version of DEER (4p-DEER), developed by Spiess and co-workers, it is possible to measure dipolar (through-space) interactions between radicals’ spins in materials and to derive information on local concentrations, distance distributions, and relative orientations between electron spins.^{43–45} In the past decade, we have developed DEER measurements on manifold albumin-based natural, artificially modified, or even albumin hybrid systems to probe the local solution structure of albumins.^{2,5–8,14} As such, we

have found that the solution structures of human and bovine serum albumin, HSA and BSA, respectively, are significantly different, while in the crystalline form they are more or less identical.^{5,14} Through solvation, HSA unlike BSA, regains conformational flexibility on the protein’s surface.

Here, we have performed DEER test measurements on one sample from the healthy control patient group and one sample from the PAC patient group. These data are presented in Figure 8. It should be noted that the dipolar evolution times of 1.5 μ s are not optimal to detect distances larger than 4 nm. This is due to the fact that unlike in samples of purified HSA, the relaxation times dominating the time frame for these measurements are shorter, so we could not reach the same time frame as in artificial *in vitro* tests with commercial HSA in buffered solutions. The reason for this is mainly due to the decreased concentration in blood samples and the strongly increasing measurement time when increasing the dipolar evolution time beyond 2 μ s. A detailed discussion on the experimental necessities is also given in refs 2 and 5.

When inspecting the data in Figure 8, in particular the center panels and the right-hand distance distributions, it is obvious that there are only minute differences at loading ratios 1:2 and 1:4 between the distribution of 16-DSA in albumin from the PAC patient sample and the healthy individual. Remarkably, all time traces are also very similar to what is found for purified HSA in buffered solution.^{2,5} At this point, we can thus only speculate that in line with the detectable (but subtle, one may add) changes in CW-EPR spectroscopy that we described in detail above, we can conclude that again large-scale conformational changes in HSA of PAC patients cannot be found for this examined sample.

Furthermore, from the modulation depths, i.e., the minimum values attained in the background corrected DEER data in the center panels, one can extract the number of interacting spins (and hence the number of HSA-bound 16-DSA molecules) in the systems. It is indeed found to be identical for both samples and is in line with what is expected for mainly dimers (at the 1:2 loading ratio) and multimers (at the 1:4 loading ratio).^{5,46,47} In particular, for the latter samples containing on average 4 FAs per HSA, multispin effects complicate the data analysis, as we have shown before,⁴⁸ and will in future studies be minimized by use of spin dilution.^{5,48} The identical modulation depths indicate that also competitive binding (or expulsion) of FAs by PAC-released molecules cannot be detected.

All the CW-EPR and DEER spectroscopic data together allow a preliminary molecular interpretation of the origin of the clearly observable and detectable changes in CW-EPR spectroscopic parameters for which we see great potential for translation into clinical applications as a screening test. Large-scale conformational changes that either reduce the number of FA binding sites (which are good indicators of the overall HSA solution structure; see the CW-EPR and DEER data) or that change the distribution of binding sites (see the DEER data) can tentatively be excluded. Yet, we do see slight changes at the protein's surface (where 16-DSA has the DOXYL group) in PAC patients that make the strong immobilization (A) binding sites more polar/hydrated. Along with this, we observe slightly increased rotational motion at these chain ends of the stearic acid derivatives, which could also be a consequence of the larger hydration at these entry points to the FA-binding sites. Hence, the PAC-induced change could be due to either small post-translational (chemical) modifications that increase hydrophilicity at certain amino acid side chains or, which we deem more probable, noncovalent interaction of small amphiphilic molecules that are known to be attachable on amphiphilic/hydrophobic patches on HSA's surface¹⁴ and that lead to small changes of hydration on the surface of the protein. As described above, this is highly speculative at this point, and further measurements on larger numbers of patient samples (CW-EPR and DEER) are necessary for allowing more robust insights into the unequivocally existing and relevant changes that are the basis of this screening test.

■ CONCLUSIONS AND OUTLOOK

Taken together, our study reveals that CW-EPR spectroscopy may be suitable for identifying patients with PAC in a screening blood test. To the best of our knowledge, the present work is the first study which examined FA-binding capacities of HSA in distinct pancreatic entities. We clearly show that especially at high loading ratios of FAs to HSA (1:4 or 1:6 HSA:FA) the EPR spectroscopic parameters $2A'_{zz}$ versus τ_c when plotted against each other in scatter plots allow discrimination of parameter regions typical for PAC patient samples. Furthermore, interpreting the CW-EPR spectroscopic data and preliminary nanoscale distance measurements using DEER spectroscopy, we tentatively interpret the molecular origin of the distinction between PAC, BPD, and healthy individuals to be due to small changes on the protein surface, in particular the hydration state at the FA binding site entry points. These changes in hydration could either stem from post-translational modifications or noncovalent attachment of PAC-released molecules that transiently attach to the protein's

surface. Large-scale conformational changes cannot be interpreted from our data at hand.

For the final translation of the screening test into clinical application, however, the examined patient numbers preferably should be higher also to increase the accuracy of the applied method. Further studies are demanded, especially in terms of validating the present results and in evaluation of postoperative EPR spectroscopy after curative surgery.

■ MATERIALS AND METHODS

Patients. All patients provided their consent for participation in this study. The informed consent was obtained according to an ethical permission (Ethics Commission Permit of the Medical Association Nordrhein, Germany, No. 2015076). The patient groups in the present study consisted of healthy individuals ($n = 24$; 11 male, 13 female), patients with BPD ($n = 26$; 11 male, 15 female) as CP ($n = 14$) or IPMN ($n = 12$), and patients with PAC ($n = 35$; 18 male, 17 female) (UICC stages I–IV).

Patient recruitment was based on results from CT and/or MRI or ERCP. Inclusion criteria for patients were the identification of a pancreatic pathology by the above-mentioned imaging modalities and the absence of a recurrent tumor, a second malignancy or neoadjuvant therapy. "Healthy control group" patients included patients presenting for elective surgery of hernia repair. They had no systemic diseases, inflammations, or tumor entities. The nutritional state of all recruited patients was documented by a standardized nutritional score.⁵⁰ Patients with BPD were either observed (small IPMNs) or operated by pancreaticoduodenectomy or pancreatic tail resection or pancreatectomy.

Operations on patients suspicious for malignancy included the pylorus preserving pancreaticoduodenectomy (PPPD), the pancreatectomy, or the pancreatic tail resection, all with oncologic lymphadenectomy. In two cases of suspected pulmonary metastases of PAC, a video-assisted thoracoscopy (VATS) with segment resection occurred. In other cases of metastasized disease, a diagnostic laparoscopy with biopsy or a palliative gastroenterostomy with or without biliodigestive anastomosis was performed. The study population was a consecutive series of participants defined by the above-mentioned selection criteria. The median ages were as follows: individuals of the healthy group, 69 years; patients with BPD, 65 years; and patients with PAC, 73 years. For further details, see Table S1. Blood samples were taken pretherapeutically by venipuncture. The time interval between sample collection and following centrifugation was 15 min. After clotting, serum was separated by centrifugation (1000 g) for 10 min at room temperature, removed, and then stored at $-20\text{ }^{\circ}\text{C}$.

Serum Sample Preparation/EPR Spectroscopy. The HSA concentration (mg per dL) of all patients was measured by titration of a standard solution of the anionic dye bromocresol green to the albumin solution until a color change (blue-green complex) could be observed photometrically. Normal values are 35–50 mg/dL. Sample preparation for EPR spectroscopy was described in detail in our study on healthy individuals¹² and is only briefly described here. All EPR spectra were recorded on a Magnetech MiniScope MS400 benchtop CW-EPR spectrometer operating at X-Band frequencies (9.43 GHz) at 298 K. A microwave power of 3.16 mW with a modulation frequency of 100 kHz, modulation amplitude of 0.1 mT, and 4096 points were used throughout the measurements. The respective final spectrum which is

subjected to analysis, is accumulated 10 times, each for 60 s, yielding a final measurement time of ten minutes per spectrum.

The spin probe, 16-DSA (16-DOXYL stearic acid), was purchased from Sigma-Aldrich (Taufkirchen, Germany) and prepared as a 26 mM stock solution by dissolving the proper amounts in 0.1 M KOH solution. Loading ratios of HSA:16-DSA were prepared from 1:1 to 1:7 in 0.5 steps (in total 12 preparations per sample). Considering a final concentration of 200 μ M of provided HSA from serum samples, proper values of serum solution were mixed with corresponding concentrations of 16-DSA in Dulbecco's phosphate-buffered saline (DPBS) buffer. Glycerol (20% vol; 87% from Thermo Fischer Scientific) was used as cryoprotectant to ensure suitable vitrification of the samples and to mimic the higher viscosity.⁵¹ The final sample volume was 200 μ L.

EPR spectra were simulated based on the spin Hamiltonian using the easyspin software package (release 5.2.25)⁵² in MATLAB version 8.6 (R2015b). The principal values of the g-tensor were chosen according to the values reported for DOXYL-labeled stearic acids in interaction with bovine serum albumin.^{53,54}

An anisotropically rotating three-component system was considered for simulations, as detailed before.^{12,55,56} To reduce complexity but to still have one value as a measure of rotational mobility, the reported rotational correlation times are calculated based on an isotropic rotational model.^{55–57} Simulation errors are calculated based on the RMS-deviations between experimental and simulated spectra for all spectral data points (4096 points). The medians of the error for all the three groups is found to be 15% for loading ratios up to 1:4 and 18% for higher loading ratios (1:5 to 1:7).

DEER Data Analysis and Instrumental Details. X-band (~ 9.4 GHz) pulsed EPR measurements were performed on a Bruker Elexsys E580 spectrometer equipped with a 3 mm Flexline split-ring resonator (ER4118X-MS3, Bruker Biospin GmbH, Karlsruhe, Germany). Sample preparation was as described above. The resonator could be overcoupled to $Q \sim 100$. The pulses were amplified by a 1 kW pulsed traveling wave tube (TWT) amplifier (Applied System Engineering, TX, Model117). Echo-detected field swept (Electron Spin Echo-ESE), T_2 relaxation measurements, and conventional 4p-DEER measurements were recorded at 50 K which is achieved by a closed-cycle cryostat (ARS-4WH, www.arscryo.com) and a Lake Shore Cryotronics temperature controller (Westerville, OH). A conventional 4p-DEER sequence was used for the measurements: $\pi/2(\text{observer}) - t_1 - \pi(\text{observer}) - t - \pi(\text{pump}) - (t_1 + t_2 - t) - \pi(\text{observer}) - t_2 - \text{echo}$.^{2,43–45}

The length of both π and $\pi/2$ pulses for the observer sequence were 32 ns to ensure for equal excitation bandwidths. The length of pump pulse was 12 ns. The initial t_1 was set to 180 ns. To suppress the nuclear modulation effects they were averaged in 8 ns steps. Time traces were recorded for a t_2 of 1.5 μ s. The pump pulse was set to the maximum of the nitroxide field sweep spectrum, while the observer frequency was set on the lower-field shoulder at offset frequency ($\Delta\nu$) ~ 62 MHz. The experiments were performed overnight, i.e., with a measurement time of ~ 12 h. DEER time traces were analyzed by the Matlab-based (release: R2016a) DEERAnalysis package developed by Jeschke and co-workers (release: 2016).⁵⁸ More details on the technique as we apply it to HSA are summarized in refs 2 and 5.

Statistical Analysis. Statistical analyses of the data were performed in MATLAB. All quantitative data are given in

terms of their median values and median absolute deviation (MAD) which both are considered robust statistics against outliers. The detailed graphical error analysis are represented in boxplots, showing the variation range, outliers, and median of the data point in each series. Considering the number of samples in this study, the Welch–Satterthwaite version of t test was applied to find the statistically meaningful differences between independent data series. The results are summarized in Table S2.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspsci.0c00116>.

More CW-EPR spectroscopic data analysis and patient group demographic/sample information (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Dariusz Hinderberger – Institute of Chemistry, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany;

orcid.org/0000-0002-6066-7099; Phone: +49-345-55

25230; Email: dariusz.hinderberger@chemie.uni-halle.de;

Fax: +49-345-55 27576

Marcos Gelos – Faculty of Health Sciences, University of Witten/Herdecke, Witten, Nordrhein-Westfalen 58448,

Germany; Phone: +49-201-8051841; Email: marcos.gelos@uni-wh.de; Fax: +49-201-8052184

Authors

Haleh H. Haeri – Institute of Chemistry, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany

Jörg Tomaszewski – Department of General and Visceral Surgery, Alfried Krupp Krankenhaus Essen, Essen, Nordrhein-Westfalen 45276, Germany

Bettina Phytides – Department of General and Visceral Surgery, Alfried Krupp Krankenhaus Essen, Essen, Nordrhein-Westfalen 45276, Germany

Heike Schimm – Institute of Chemistry, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany

Gabriela Möslein – Faculty of Health Sciences, University of Witten/Herdecke, Witten, Nordrhein-Westfalen 58448, Germany

Marco Niedergethmann – Department of General and Visceral Surgery, Alfried Krupp Krankenhaus Essen, Essen, Nordrhein-Westfalen 45276, Germany

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acspsci.0c00116>

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This research was financially supported by financial support from the state of Saxony-Anhalt (European Regional Development Fund–ERDF grant ZS/2016/06/79740).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Annkatrin Rother (MLU) for technical support.

■ ABBREVIATIONS

EPR: electron paramagnetic resonance; ESR: electron spin resonance; CW: continuous wave; DEER: double electron–electron resonance; HSA: human serum albumin; FA: fatty acid; 16-DSA: 16-DOXYL stearic acid; MAD: median absolute deviation; ERCP: endoscopic retrograde cholangio pancreatography; PTM: post-translational modifications; CT: computed tomography; MRI: magnetic resonance imaging; PAC: pancreatic adenocarcinoma; BPD: benign pancreatic disease; CP: chronic pancreatitis; IPMN: Intraductal papillary mucinous neoplasms; UICC: Union for International Cancer Control

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